

Doctrinal fallacies of stewardship

Britain's environmental minister, Mr Christopher Patten, has taken on the no-growth lobby, somehow brought to life again by novel concerns about the global environment, but has done so more cautiously than it deserves.

Mr Christopher Patten, the engaging environment minister in the British government, could yet be the prime minister if the British are ever weaned from the belief that only women can do that job, however imperfectly. Last week, he spent an uncomfortable time at the Hague, defending his government against the charge that Britain is an environmental black sheep, especially in relation to pollution of the North Sea. The complaint is largely spurious; certainly those concerned would not last week have been as clamant were Britain less mean-minded on broader European questions.

Now, as if to demonstrate that he is really on the side of the angels, not the ogres, Patten has published in *The Times* his reasons for believing that economic growth is not incompatible with what he calls "the central ethical principle" that should underlie environmental policies — that of "stewardship". High time, it may be thought, that some government's spokesman took the continuing and repetitive environmental argument onto a more exalted plane. But, sadly, although Patten says many of the right things, he does not say enough of them.

For one thing, Patten defines stewardship too narrowly: "We do not have freeholders' rights to the land we live in . . . we are trustees, obliged to pass on to the next generation what we have inherited from the last." (Why just the land?) He goes on to cite Blake and Dickens as proof that the notion is reflected in Victorian literature, points out that too many people infer from the principle of stewardship that the sum total of economic growth must be zero (or that countries now rich must be impoverished so that others may become richer) and easily derides the implications of the breathtaking statement attributed to M. Jacques Delors, the President of the European Commission, that "we must respect nature for itself and not simply as a means of satisfying our own needs: nature has a logic of its own which may differ from ours". Patten rightly declares that the extreme implication of this principle — "leave things alone unless absolutely necessary" — is plain wrong.

Altruism

The doctrine of stewardship is not, of course, new. Subversive St Augustine in the fifth century AD, persuaded by his pastoral experience of the weakness of the then conventional proposition that, for people, life on the surface of the surface of the Earth is merely an irksome postponement of life in Heaven — people seemed not to

welcome death — figured out that people's regard for their children and their children's children is a natural means by which one generation compromises its immediate interests for the sake of those that lie ahead. The modern equivalent of the augustinian imagery is W. D. Hamilton's technical definition of altruism to account for the behaviour of social insects, vividly if riskily generalized in Richard Dawkins's book *The Selfish Gene*. But one of the achievements of the intervening 15 centuries is the recognition that stewardship is not simply equated with physical (which includes biological) conservation.

By now, there are ample illustrations of that simple truth. Auks, for example, have disappeared, supplies of amber much valued by the Romans are much diminished now, as are those of emeralds, surface deposits of coal are almost gone, petroleum is a declining source of fuel whose extraction is bound to be much more expensive a century from now, the whole landscape of Europe and much of that of North America has been remodelled by post-glacial climatic change and, more recently, by agriculturalists, and molybdenum becomes more scarce with the passage of every year. On the extreme view, all these happenings are offences against the strict reading of the doctrine of stewardship. In reality, they are nothing of the kind. Losses from the physical (which includes the biological) environment have been more than compensated for by gains in social capital (which term includes not only most countries' educational systems but the world's accumulated stock of technological skill).

Pillage

Patten might have teased those who hold to the strict interpretation of stewardship by demanding a demonstration that the present generation (the likes of us) is hampered in the management of affairs — its capacity to discharge responsibilities of stewardship included — by past "unbridled pillaging . . . of the natural world", as Delors puts it. Take, for example, industrial pollution, of which there is too much. (Mischievously, Patten argues that pollution is not the necessary consequence of economic growth by noting that the horrendous pollution of Eastern Europe now coming to light is the product of economic stagnation.) Industrial accidents apart, there is no place in the world where the impairment of public health by industrial pollution outdoes the improvement of public health brought about in the past century and a half, first by the rational improvement of water supplies and,



latterly, by the practice of medicine, often rudimentary medicine.

The trick that the no-growth lobby plays on itself (and would foist on others) is to insist on the application of a kind of law of detailed balancing (*Ehrenfest*) to environmental matters: environmental costs must be outweighed by benefits of exactly the same kind. That is the route to nonsense, as is much else in the no-growth lobby's case. If, for example, the present generation, knowing as it does that the supply of low-cost petroleum will soon be exhausted, were to conclude that further exploitation should be halted, how would later generations resolve what is exactly the same dilemma? Presumably they would have no choice but to follow suit, with the consequence that a valuable (because cheap) fuel would be permanently fossilized economically, in trust for generations who could never use it. Would it not be wiser that the economic rent of this resource should contribute here and now to the more constructive purpose of winning energy from sunlight, which should be available at least until the Sun becomes a red giant?

That, broadly speaking, is what past "unbridled pillaging" has accomplished. Some natural resources have gone for good, but have been used in part to generate the skill with which they can be replaced, naturally or by synthetics. Some parts of the world have allowed pollution to get the better of them, but the technology from which the pollution springs is both the means of understanding how it may be removed and the means of generating the economic resources without which the task will never be attempted. Some species have been exterminated, but not those whose disappearance would threaten human survival, which have instead been made to flourish remarkably by the efforts of agriculturalists, plant and animal breeders for example. None of this is to suggest that everything that has happened in the past has been for the best in the best of all possible worlds, but merely that the present generation and, by extension, its successors, are fortunate that events have turned out as they have.

Luck

But can the luck hold? That is what the no-growth lobby asks. The question is only as meaningful as the premise that only good luck has brought us this far. The reality is different. The transformation from pastoral to agrarian society was founded on the calculation that the nutritional benefits of growing crop plants would so far outweigh the then unforeseen disadvantages of village life that the security of tradition might be safely abandoned.

Now, we are more self-conscious. Vaccinia vaccination was welcomed in the late eighteenth century by its recipients and their parents, the then great risks notwithstanding. The inclinations of pell-mell industrialists in the nineteenth century were quickly restrained by legislation, often needlessly so (as when men with red flags walked ahead of railway trains). Now, even governments have become used to plotting strategies for the future in the design of which broad environmental issues are near the

surface. Why else have industrial governments embarked on century-long research programmes directed at the generation of thermonuclear energy? And have accepted, however reluctantly, that rich countries have some kind of obligation towards the poor?

Yet, the no-growth lobby says, no amount of prudence can forfend the unique danger arising because the scale of the human enterprise is now comparable with that of the geophysical processes that shape the physical environment. First there was the threat of nuclear war, now there are chlorofluorohydrocarbons that threaten to get rid of ozone in the stratosphere and, beyond them, the prospect of a damaging excess greenhouse in the lower atmosphere. Are they not proofs that security in the future is a thing of the past?

Patten's rejoinder that "sound science" is the answer would be more convincing if the government of which he is a member had been more imaginative in its support of its own endogenous research enterprise, but the principle is sound enough, although only part of the truth. The difficulty, as Patten's government keeps saying, is that sound science costs money and, in the environment of domestic politics — every bit as tangible as the physical environment — can be sustained only by economic growth. Those who believe otherwise should glance at the United States, where a now-popular government can think of ridding itself of a budget deficit that cramps its freedom only by growing so fast that the tax-yield will grow faster than the government's obligations. It is not that those who elected President George Bush to office are indifferent to their children's future, but that the surest way of helping them quickly to act altruistically (in Hamilton's sense) is to let them keep the appearances of prosperity.

There is a wider truth to learn. The decades immediately ahead will be thieves of the industrial world's economic resources. Starting now, resources (in the tangible form of manufactured capital goods) will have to flow to Eastern Europe and the Soviet Union on an unprecedented scale if expectations of newly acquired personal liberty are not to be dangerously frustrated. If (as this magazine has advocated) there is a convention on greenhouse gases, only dizzying economic growth will provide the resources with which to replace the generating plants now in being. Beyond that are problems such as Africa, which, even before AIDS, seemed destined to be the first continent to be lost to human habitation through wayward mismanagement.

These are the issues with which the no-growth lobby should be confronted. As in the lobby's first flowering, in the early 1970s, the view that the trouble with the world is growth, stems from a profound misunderstanding of what the world has been for the past 5,000 years, from ignorance of ideas as different (or, rather, as alike) as those of St Augustine, Adam Smith and W. D. Hamilton and from misanthropy. It is fortunately belied by most of history and by the now widely shared sense that a changed world provides hitherto foreclosed opportunities. □

Faltering steps towards European/US cooperation

- Commission vice-president jumps the gun
- IBM feeling unwanted in Europe

Washington

IN an announcement that left both US and the European Communities (EC) officials at a loss for details, the EC's commissioner for research said last week that he had called on the White House to join in plans for a new US-EC science task force. EC commission vice-president Filippo Pandolfi told participants at a two-day meeting at the US National Academy of Science on EC science that he had asked the President's Science Advisor, D. Allan Bromley, to consider establishing a permanent joint committee to set standards for scientific cooperation between the United States and the EC.

Bromley said he had "agreed to look into" the proposal, but pointed out that he has already set up his own advisory structure to coordinate cooperation between the United States and the EC. Because of the different and sometimes contradictory ways that Europe and the United States run science, Bromley said he "suspects that we will end up with slightly different mechanisms on both sides of the Atlantic". Soon after taking office last summer, Bromley decided to revamp the Federal Coordinating Council on Science, Engineering, and Technology (FCCSET) to include, among other things, an inter-agency working group on EC science and technology issues. Although he did not shut the door on the possibility of a joint task force, Bromley emphasized that he was counting on his own advisory and policy-setting groups to "play an important role in developing an effective bridge" between the United States and the Communities.

EC officials generally distanced themselves from Pandolfi's initiative. Gilbert Fayl, the EC's science and technology counsellor in Washington, says that Pandolfi "expressed his own views" in offering the proposal and was not necessarily reflecting EC policy. The possibility of formal cooperation has come up in the past, but is not clear if the EC have been given the authority by the members to make such an agreement.

Other issues discussed at the academy meeting included the impact of the changes in Eastern Europe on EC programmes. EC officials say that rebuilding the "science infrastructure" will be a substantial part of an evolving initiative known as the Poland-Hungary Assistance for Economic Restructuring (PHARE) which includes economic stabilization support of \$1,000 million to each of the

two countries.

Bromley said that several Eastern European science delegations have recently visited the White House to review current scientific agreements. But he stressed that significant scientific exchanges between the United States and Eastern Europe must still cross a cultural and economic divide.

The movement of scientists and technology between East and West is expected to throw off the balance of many EC programmes in the coming years. Many researchers are already abandoning the neglected universities of Eastern Europe in favour of Western institutions. Meanwhile, EC nations are gearing up to ship industrial technology to the newly democratized Eastern European nations. The consequence of more researchers competing for scarce basic science resources in the West, while increasing amounts of money move East, is that EC science may be hard-pressed to show any real gains in the near future, says National Science Foundation analyst Richard Bradshaw. But although EC-supported research accounts for only four per cent of the total research and development spending of the EC nations, the spending is "leveraged" by its concentration on consortia and collaborative ventures where a modest investment allows access to broad areas of state-of-the-art research. In microelectronics especially, EC projects are becoming increasingly attractive to US companies, according to Bradshaw.

To get full, equal-partner access to EC-supported research, a US company must have established manufacturing, marketing, and research and development operations within the European Communities. So far, it has been primarily US electronics companies that have made that commitment.

But at the academy meeting, Jean-Jacques Duby, science and technology director for IBM-European, complained that meeting the criteria has not translated into equal-partner status for IBM.

Duby said that although IBM is now allowed to compete for access to projects, it finds that many of its proposals are being rejected. "The EC asks outside experts to decide on proposals, but most of them come from European-held companies, and none come from IBM", he said. Foreign-owned companies have "slightly less equal rights" to participate in EC research, he claimed.

G. Christopher Anderson

FUSION RESEARCH

New panel to decide future of US programme

Washington

THE US fusion research programme, which includes both traditional 'tokamak' reactor designs and more novel laser-driven 'inertial confinement' devices, is to be re-evaluated by an advisory panel set up last week by the Department of Energy (DOE). The panel is expected offer its advice on recent controversial attempts to shift the balance between research on the two techniques.

Former DOE energy research director Robert Hunter caused trouble in Congress and the fusion physics community last year by proposing to shift money from the magnetic fusion programme in order to accelerate research into laser-driven inertial-confinement designs, which evolved out of nuclear weapons research. Hunter's move was angrily interpreted in Congress as an attempt to change national policy without proper consultation (see *Nature* 341, 476; 1989). James Decker, now acting energy research director in Hunter's place, assured a House appropriations subcommittee last week that DOE secretary has an "open mind" on the future of fusion research, and "is not married to the Hunter plan". H. Guyford Steyer, who has served as both presidential science adviser and director of the National Science Foundation, will chair the DOE panel. For the time being, Decker told the congressional committee, the present balance between magnetic and inertial-confinement would be maintained. But when the DOE review was completed in September this year, the 1991 fusion budget would "be revised as appropriate," Decker said.

Last month a National Academy of Sciences (NAS) panel released an interim report on the DOE programme that recommends against the immediate pursuit of energy production with laser-driven fusion techniques. The NAS report, produced at the request of Congress, is a review only of the technical progress and prospects of the US fusion effort; the DOE panel, on the other hand, is being asked to make policy recommendations based on economic, political, and scientific considerations.

The NAS panel believes that inertial confinement techniques have a long way to go before they can form the basis of an energy-production technology, and therefore encourages DOE to maintain laser fusion research as part of a weapons programme. Although that conclusion would seem to discourage DOE from running a direct competition between inertial-confinement and magnetic fusion to find the most promising approach to energy production - the strategy Hunter had proposed - the NAS panel stopped short of making a comparison between the programmes.

G. Christopher Anderson

NASA denies negligence

Washington

CLAIMS that the National Aeronautics and Space Administration (NASA) has, through neglect and inaction, lost valuable data from early space missions have been dismissed by the agency as old news. NASA officials concede that storage of magnetic data tapes at a number of facilities falls below federal standards, but deny that any significant amount of information has been either lost or irretrievably damaged, and say that efforts to assemble a comprehensive data archive are well in hand. But some space scientists agree that data management has not been one of NASA's strengths, and say that the current programme of data restoration is due largely to the initiative of the research community.

The criticism of NASA comes from the US General Accounting Office, an investigative body of the Congress, in a report issued last week*. The bulk of the report claims that NASA's storage facilities for magnetic tapes are substandard. Only two out of ten centres (the Smithsonian Astrophysical Observatory in Cambridge, Massachusetts, and the Center for Astrophysics and Space Sciences at the University of California in San Diego) received GAO's partial approval; the other facilities failed to meet two or more federal tape-storage standards. GAO's evidence includes photographs of tapes stacked in hallways, packed in cardboard boxes rather than hung on racks and stored near air-conditioning units and in damp basements. Also, says the GAO report, many tapes have been untouched for years, contravening requirements that they should be regularly rewound to prevent magnetic 'print-through' of data between adjacent layers of tape.

NASA's official response to the GAO report accepts most of these charges, but points out that no specific evidence is given of tapes having been lost or become unreadable. At the Jet Propulsion Laboratory (JPL) in Pasadena, California, where much of the data from early planetary explorations is stored, recent attempts to transfer data from old tapes have been 99 per cent successful. Art Zygielbaum, manager of the Science Information Systems Office at JPL, denied the GAO's allegation that half of 135,000 old tapes in his charge might be useless; he says that of 35,000 examined so far, 90 per cent contained useful, and readable, material and the remainder were back-up or duplicate copies.

JPL's 'data salvation' programme was cited by Ray Arvidson, of the McDonnell Space Sciences Centre at Washington University in St Louis, as proof that

GAO's criticism is "behind the times".

The JPL programme is part of the Planetary Data Systems project (PDS), begun in the mid-1980s on the advice of scientists who constituted the Committee on Data Management and Computation (CODMAC), of which Arvidson is a past chairman.

CODMAC was in turn formed in 1978 on the advice of the Space Sciences Board of the National Academy of Sciences.

The success of PDS, says Arvidson, hinges on its direct involvement in data archiving of the scientists who need the data for their research. As PDS progresses, JPL will transfer planetary data to compact disks, and complete copies of the archive will be given to several interested institutions, including the McDonnell Centre.

The biggest stumbling block in PDS, according to Arvidson, is not the technical matter of getting data off old tapes, which has been highly successful, but the archival problem of working out what the data in hand actually represent. In early missions such as Viking and Mariner, much of the scheduling was worked out 'with pencil and paper' as the mission proceeded: data were recorded, but frequently the mission logbooks were cast aside in the rush of events. The hasty and improvisatory pace of these missions was not conducive, says Arvidson, to good data management.

Nevertheless, data from planetary missions appear to be generally safe. The same cannot be said of other areas, according to Chris Russell, a planetary geologist at the University of California at Los Angeles who was chairman of CODMAC after Arvidson. In the early 1980s, CODMAC made recommendations to all the departments of NASA with responsibility for data, and the Office of Space Science and Applications proposed the PDS project. But in Earth sciences little was done; Russell says that NASA scientists felt that the data were in "such a bad mess" that they may have been irretrievable. Although some committees and workshops were set up to study the problem, Russell says there was a general feeling that it was better to concentrate on new missions that would bring better data. In planetary research, by contrast, there were few assurances that new missions would ever come about, and the existing data were therefore more highly prized.

But with concern over the global environment having led NASA to embark on its comprehensive Earth Observing System (EOS), old data, even if of poor quality, take on new importance for the investigation of long-term global trends. The National Space Science Data Center, at the NASA/Goddard Space Flight

Japan looking for a profitable solution

Tokyo

THE curiously named Institute of Industrial Technology for the Global Environment — Japan's practical response to worries about global climate change — is to be located in Kansai Science City, near Osaka, and should be in business early in 1992, according to an announcement made last week. The institute, set up with massive financial support from Japan's Ministry of International Trade and Industry (MITI), will work on chlorofluorocarbon substitutes, biodegradable plastics, new biologically based production processes that consume little energy, and technology for converting carbon dioxide emissions from industrial sources into useful products, such as methanol.

Attempts will be made to convert carbon dioxide both by conventional catalytic hydrogenation and by a more fanciful approach using vast vats of photosynthetic microorganisms or algae fed with concentrated sunlight delivered through optical fibre cables. These and related projects are the subject of a ¥6,010 million (\$43 million) a year programme beginning in 1990 and expected to continue for 10 years.

Until the institute opens, research will be carried out at other MITI laboratories and in private industry. Chemical engineering and biotechnology companies will provide additional financial support and most of the 80 or so researchers who will work at the institute. David Swinbanks

Center in Maryland, is now embarked on an agency-wide effort to transfer all important data to optical disks. But too little has so far been, says the GAO report, for the ultimate success of this programme to be judged.

In its response to the criticisms, NASA emphasizes its plans to look after the data from current and imminent missions. In the past, according to NASA spokesman Charles Redmond, ten per cent of the cost of every mission was set aside for 'mission operations and data analysis', but for EOS the figure for data management alone is set at 35 per cent. A similar commitment has been made for the Hubble Space Telescope.

Arvidson and Russell agree that NASA is doing a better job now than in the early days, when the agency's success was measured by the number of new missions carried out and safeguarding data won promotion for nobody. In 1988, the National Academy of Sciences decided CODMAC was no longer needed, and despite Russell's feeling that it still had a purpose in reviewing the products of NASA's new-found diligence, the committee was disbanded. Now, as before, it is up to individual researchers to look over NASA's shoulder. David Lindley

* NASA is not properly safeguarding valuable data from past mission, US General Accounting Office IMTEC-90-1.

BIOTECHNOLOGY INDUSTRY

Streptokinase equal to TPA

Washington

RESULTS from an Italian study comparing the blood-clot dissolving agents tissue plasminogen activator (TPA) and streptokinase show that TPA is no more effective than streptokinase, which is one-tenth of the price and widely used in Europe. Biotechnology industry analysts are worried that sales of the TPA product Activase, which last year brought in \$196.4 million for the San-Francisco-based Genentech, may be hit by the news.

The study, sponsored by the National Association of Cardiologists and the Mario Negri Institute of Pharmacological Research in Milan, was based on the treatment of 20,891 heart-attack victims.

Results reported last week at a meeting of cardiologists in Florence, are derived from an analysis of only the 12,381 patients involved in the Italian half of the study.

The patients were randomly divided into two groups and given intravenous infusion of either TPA or streptokinase. Half of the patients in each of the two groups also received a subcutaneous injection of heparin 12 hours after administration of the thrombolytic agent. Following standard medical practice, aspirin and beta blockers were also administered. The mortality rates when TPA and streptokinase were given alone were 8.7 and 9.2 per cent, and in combination with heparin the rates were 9.2 and 7.9 per cent. TPA caused fewer allergic reactions, less hypotension and fewer bleeding episodes, but streptokinase patients suffered fewer subsequent strokes.

For reasons of cost alone, these findings could significantly alter US medical practice, which at present favours TPA.

Genentech is the only US company with approval to sell a recombinant version of TPA, and its greatest competition comes from streptokinase, which is derived from streptococcal bacteria and sold in the United States by Hoechst-Roussel Pharmaceuticals and SmithKline Beecham. Activase, which costs \$2,200 per dose, now commands a two-thirds share of the US thrombolytic market.

Genentech claims that the significance of the Italian study may be reduced because it did not use the treatment favoured by "98 per cent of US cardiologists", whereby intravenous heparin is given concurrently with the thrombolytic agent.

San Francisco stock analyst John Girton says the results of the trial make it "harder to justify the price of TPA", but he believes that TPA is still a healthy proposition, and points out that it may have other uses. Indeed, Genentech is awaiting approval of Activase for pulmonary embolism, and is conducting clinical trials for the treatment of both unstable angina and occlusive stroke.

Girton does not foresee any impact on the Genentech agreement, where Roche will acquire a 60 per cent stake in Genentech in return for a \$2,100-million investment. But Stuart Weisbrod of Prudential-Bache takes a gloomier view and predicts that the growth in sales of Activase will slow from 25 to 30 per cent a year to 5 per cent.

Diane Gershon

VETERINARY SCIENCE

Antelopes die of "mad cow" disease

London

SOME zoo animals may have been infected with the agent which causes bovine spongiform encephalopathy (BSE), the disease which has killed over 10,000 British cattle. Five antelopes have died in British zoos from a BSE-like condition, thought to have been transmitted in commercial feed containing sheep meat infected with scrapie, a similar disease. Although this practice has been stopped, the long incubation period of spongiform encephalopathies means it may be several years before the extent of infection in zoo animals is known. James Kirkwood, senior veterinary officer at London Zoo, where an Arabian oryx and a kudu died last year, says that conservationists will have to wait to assess the impact of the outbreak on endangered species.

London Zoo officials have written to other British zoos suggesting that "careful consideration" should be given before animals at risk are exported to foreign zoos or for reintroduction programmes. London Zoo's circular says that any ungulate fed

proprietary meal between 1980 and 1989 may be affected.

The first recorded case of the disease in a zoo was in 1986, the first year of the outbreak of BSE in cattle, when a nyala at Marwell Zoo, near Winchester, died. Since then, in addition to the London cases, a gemsbok has died at Marwell, and an eland at Port Lympne Zoo, in Kent. The cross-species transmission of spongiform encephalopathies is not a surprise; results published in the *Veterinary Record* in February showed that laboratory mice can contract BSE symptoms after eating infected cattle brain tissue.

Geoff Holmes, from the agriculture ministry's veterinary centre at Ithchen Abbas, where the Marwell cases were examined, says his biggest worry is whether the disease will be passed from female cattle and antelope to their offspring, via the placenta. This does occur in scrapie-infected ewes, but research to test for this 'vertical transmission' in cattle is still in progress.

Peter Aldhous

COMPOUND Q

AIDS drug trial resumes

Washington

THE controversial study of the AIDS drug Compound Q, which was halted last August by the US Food and Drug Administration (FDA), is to be resumed with full FDA approval. It is the first time the FDA has granted an independent IND (Investigational New Drug) licence to a community-based research group.

News of the decision will enrage many doctors and researchers who feel that the original 'clandestine' study, coordinated by the San Francisco-based advocacy group Project Inform, was poorly designed, gave inconclusive results and may have caused unnecessary suffering to patients (*Nature* 341, 267; 28 September 1989). The *ad hoc* study began last May after *in vitro* experiments showed that Compound Q, a protein (trichosanthin) derived from a Chinese cucumber-like plant, selectively killed cells infected with HIV.

Sandoz Pharmaceuticals of East Hanover, New Jersey is providing Project Inform with \$250,000 in financial aid, stipulating that the grant is for the retreatment of patients who participated in the unauthorized study last year. In addition, Genelabs of Redwood City, California is donating a highly purified form of Compound Q called GLQ223.

The FDA's decision to approve the trial has caused Donald Abrams, an AIDS researcher at the San Francisco General Hospital, to reconsider his position on the agency's antiviral drugs advisory committee. He thinks that the FDA should have consulted with the advisory committee in view of the atypical nature of the new clinical trial and the controversy that surrounds the original unauthorized trial. He believes it sets a bad precedent, as the same people who performed an unauthorized trial have now received approval from FDA.

The new study is designed to evaluate the safety and effectiveness of GLQ223 over the longer term. GLQ223 will be administered to over 100 patients in San Francisco, Los Angeles, Miami and Florida, all of whom will have previously been treated with the Chinese version of the drug. Patients will be assigned randomly to two groups, one receiving treatment every three weeks, the other every six weeks, while being allowed to continue with other AIDS therapies.

Genelabs, who holds the patent rights for GLQ223 as a possible treatment for HIV infection, ARC and AIDS, is evaluating the drug in its own FDA-approved clinical trials. Preliminary findings of phase I trials are expected to be submitted for FDA review in mid-1990.

Diane Gershon

US response to Sellafield data

Washington

THE startling suggestion in a recent UK study that low-level radiation doses to men may cause genetic defects in their children has sparked a flurry of calls for independent verification in the United States. First results of a US study could be out early this summer.

A team led by Martin Gardner of the University of Southampton last month released results showing that children whose fathers worked at the Sellafield nuclear waste reprocessing plant were seven or eight times more likely to develop leukaemia if their fathers had received a total radiation dose of 100 millisieverts, or over 10 mSv in the six months before conception (*Nature* 343, 679; 22 February 1990). A similar study, with results due out in June, is being conducted by John Boice of the US National Cancer Institute (NCI). Boice and his colleagues surveyed cancer deaths in 113 counties containing or adjacent to 61 US nuclear facilities. All 52 commercial nuclear power facilities that started operation before 1982 are included, as are nine Department of Energy (DOE) facilities.

The nationwide study was begun in 1987 because of general public health concern, amplified by a British survey of cancer deaths near UK nuclear facilities that showed 'clusters' of childhood leukaemia (*Nature* 329, 499; 1987). DOE facilities were included in the NCI survey because they are similar to the UK nuclear plants.

Although the US study concentrates on children living near nuclear plants, whether their fathers work at the facilities or not, further analysis of the data by parental occupation should be able to reveal a 'Sellafield effect'. Boice will not comment on the UK study or his team's results until the NCI study is released.

Elsewhere in the United States, the Sellafield study is expected to prompt new research into the possibility of inherited genetic damage.

A special session at the American Statistical Association's August meeting in Anaheim, California will discuss the Sellafield results and how best to assess the effects of low-dose radiation.

US researchers say the Sellafield results, if confirmed, are surprising enough to change dramatically the science of radiation epidemiology. "The fact that the radiation exposure to fathers [in the study] was within guidelines, and that those [small] doses are capable of making changes in the reproductive cells is biologically difficult to explain", says Shirley Fry, an epidemiologist at Oak Ridge Associated Universities, who is studying workers at DOE facilities. Although "the reaction has been substantial on this side of the Atlantic", says Daniel Hoffman,

assistant director of the Center of Environmental Health at the Centers for Disease Control in Atlanta, "the main thing now is to get replication with the large cohort of DOE and [US] nuclear plant workers". He says that although there have been weak associations between childhood leukaemia and paternal radiation doses in previous US surveys, the Sellafield study is the first to make a convincing case.

The Sellafield study, however, comes at an awkward time for the DOE epidemiology programme. In an interim report submitted last week, a DOE advisory panel recommended that much of the agency's research into the health effects of radiation exposure should be transferred to other agencies to avoid the widespread perception of conflict of interest (see *Nature* 344, 92; 8 March 1990). Although the panel has also called for increased funding for epidemiological research at the agency, director Robert Goldsmith says "it is not clear what is going to happen" to the programme.

G. Christopher Anderson

■ Reports from India allege that leukaemia clusters have been found near nuclear installations, page 185.

PUBLIC HEALTH

More radiation hazards

London

A REPORT published last week suggests that many workers in non-nuclear heavy industries may have "potential problems" similar to those caused by radiation exposure in the nuclear industry. The suggestion, from Professor Murdoch Baxter and colleagues at the Scottish Universities Research and Reactor Centre, has been widely publicized in the wake of the Gardner report, which linked childhood leukaemia near the Sellafield nuclear reprocessing plant to fathers' radiation exposure (*Nature* 343, 679, 1990).

But the British government's radiation watchdog, the National Radiological Protection Board (NRPB), has rejected Baxter's conclusions.

Baxter was commissioned by the East Yorkshire Health Authority to investigate the possibility of a link between a cluster of childhood cancers on Humberside and discharges of radioactive polonium from the Capper Pass tin-smelting plant. Baxter found no evidence that radioactive discharges are to blame, but his report says nevertheless that the concentrations of a number of radioactive nuclides in the ore processed at the plant are potentially hazardous. Working practices at Capper Pass and elsewhere "should be brought into line with those which apply through-

Decline in R&D expenditure

Cape Town

EXPENDITURE on research and development in South Africa declined from 0.96 per cent of gross domestic product in 1985-86, to 0.88 per cent (R1,329 million) in 1987-88, according to a report from the Department of National Education. The private sector share of spending has remained almost constant over the two-year period at 41.3 per cent but has declined since 1983 when it stood at 51.2 per cent.

The tertiary education sector receives 25.5 per cent of total research and development funds, considerably more than in most OECD (Organization for Economic Cooperation and Development) countries and indicating the sector's crucial role in South Africa's economic development. Universities receive 98.2 per cent of these funds, with the University of Pretoria receiving the highest proportion (14.6 per cent), followed closely by the University of Cape Town (14.5 per cent).

Of government spending, a massive 26 per cent goes to agriculture against two per cent for the United States and West Germany. As a matter of policy, the report does not indicate the percentage of funds going to military research and development.

Michael Cherry

out the nuclear industry", the report concludes.

John Hipkin, from NRPB's office in Leeds, agrees that there is some radioactivity in the ore. But radiation doses received, he says, depend on the dustiness of the atmosphere in the plant and the timing of workers' exposure. NRPB already monitors working practices at Capper Pass and similar sites, Hipkin says, and where there are problems, ventilation is improved or workers wear dust masks. Hipkin charges that Baxter's reference to Gardner's findings is "completely irrelevant" because radiation doses in Capper Pass are many times less than those that were measured at Sellafield. Michael Snee, a radiotherapist who was an author of the Gardner report, agrees that very few workers outside the nuclear industry receive radiation doses comparable to the Sellafield levels, but thinks may be some exceptional cases, such as industrial radiographers who use radioisotopes to trace leaks in metal pipes.

Peter Aldhous

Correction: The 22 February news story about the Gardner report stated incorrectly that the nuclear reprocessing plant at Dounreay is operated by British Nuclear Fuels. In fact the site operators are the UK Atomic Energy Authority.

Martian flotilla prepares

Washington

WITH a huge amount of luck, and an equal amount of hard work, 1992 will see an international fleet of tiny spacecraft, each with gigantic sails of aluminized plastic, set off on a sailing race across space. The winner will be the first to ride to Mars on the wind of photons from the Sun.

The race should take two to three years, according to Klaus P. Heiss, chairman of the group is organizing the race as one of the Christopher Columbus Quincentenary Jubilee Commission's official events. Over half the voyage time is needed to gain speed to break out of the high Earth orbit from which the craft will be launched. Then it's a year of plain sailing to Mars.

There were to have been three craft, representing America, Europe and Asia and named Nina, Pinta and Santa Maria after Columbus's ships, and the three winning designs should have been selected last year. But the schedule is behind and the number of good designs large. Britain, the Soviet Union, China and Italy have all submitted proposals, as have three US teams and a Canadian team. An Israeli group is expected to join in.

Heiss now talks optimistically of more than three craft going into space, and team representatives from Canada, Johns Hopkins University, Massachusetts Institute of Technology (MIT), Britain and Italy all say they can probably find ways to raise the \$5–10 million needed to build their sail craft. But getting the craft into space in another matter. A single launch into Earth orbit could cost \$10–100 million, and all contenders are looking to Heiss to raise the money.

Heiss says he has one "US launch commitment to geosynchronous orbit", but he cannot yet reveal the identity of the backer. He also says that the Italians will be able to launch their craft on an Ariane rocket, but a representative of the Italian team at the aerospace manufacturer Aeritalia could not confirm Heiss's statement. He did say, however, that an announcement about the race can be expected next week at a space commerce congress in Montreux, Switzerland.

All the sail craft designs owe something to the past. The idea has come up often in science fiction novels (Arthur C. Clarke's *The Wind from the Sun* was a classic of the genre), and the National Aeronautics and Space Administration (NASA) paid for a design study in the 1960s. Plans were made in the 1970s to sail out to meet Halley's comet.

The race craft have to be light and be able to deploy large sails (up to 10,000 m²) from a tiny volume. Most of them end up looking like umbrellas or multi-rayed sunflowers — carried to an extreme in the Johns Hopkins University design that has 480 'petals' which unroll during deployment. The British craft, designed by a team at Cambridge Consultants, looks more like a gigantic flying chapati or pizza, though its designers describe it as a "space manta ray". The sails tow tiny craft equipped with sensors, communication gear and steering lines. The designs differ most in how the sails are unfurled and kept extended.

The Canadian Space Society's craft is a little different, being a cross between an umbrella and a venetian blind. Its hexagonal sail is divided into thin strips and the craft can be steered by changing their pitch angle.

The "gadfly" in the fleet, as Heiss refers to it, is the radical design from Andreas von Flotow at MIT. His "helio-gyro" weighs a mere 20 kg, less than a tenth the weight of most of the others, and looks like a windmill with eight long arms radiating from a central core. Each of the arms is made from plastic film and can simply be rolled up for compact storage during launch. Once in space, the craft will be spun on its axis by a small rocket so that centrifugal force unwinds the arms and keep them rigid.

Small changes in the pitch of the blades will alter the direction in which it is blown. The craft is incredibly simple — mechanical devices (which are often unreliable) are not needed to deploy the arms and such tiny forces are need to control their pitch that motors can be replaced by piezoelectric films.

Alun Anderson

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

TWENTY-FIVE YEARS OF OBSCURITY: The US Air Force's still-secret SR-71 Blackbird spy-plane 'retired' in a blaze of glory last week, breaking speed and distance records by flying for 2,300 miles (3,680 km) between Los Angeles and Washington in 64 minutes 5 seconds. The aircraft holds the absolute air speed record at 2,193 m.p.h. (over a measured course) and altitude record of 85,069 feet (AP).

Brazil cuts red tape

São Paulo

BARELY a week before a new federal government was due to assume power, the Brazilian Ministry of Science and Technology (MCT) finally gave in to more than 15 years of appeals from scientists and agreed to reduce restrictions on the import of scientific equipment and lab materials.

The new legislation is an enormous improvement, according to Fernando Galembeck, secretary-general of the Brazilian Society for the Progress of Science (SBPC), but there remain problems with its form. The government has issued only a *medida provisória* (provisional measure), which does not have the same status as a law passed through Congress and will cease to have effect unless it is reissued or turned into a normal law. That will be the responsibility of the new government of president Fernando Collor de Mello, who assumes power this week.

Collor is sure to back a new law, according to Antonio Paes de Carvalho, secretary-general of the Bio-Rio foundation. Paes de Carvalho is an advisor to Collor and may be chosen to head the new Secretariat of Science and Technology.

Scientists have had enormous difficulties in importing materials as an import worth a few hundred dollars faced the same set of delays, taxes and labyrinthine regulations as multi-million dollar imports by giant corporations. The new measure exempts scientists from import taxes and concentrates responsibility in the MCT's National Council for Scientific and Technological Development (CNPq).

A quota for imports will be set each year by the Ministry of Finance and CNPq. The Minister of Science and Technology, Decio Leal de Zagottis, says that the quota amounts to US\$80 million per year.

Ricardo Bonalume

NUCLEAR FUELS

Allegations of laxity

Bangalore

IN response to growing numbers of reports of birth deformities, leukaemia and bone diseases among people living in the vicinity of the India's Nuclear Fuels Complex (NFC), on the outskirts of Hyderabad, company officials are maintaining that radiation exposure in the plant has never exceeded permissible limits. But a study by a team from the Bhabha Atomic Research Centre has shown that nitrate and fluoride levels in wells near the NFC are much higher than the safe levels given by the World Health Organization for drinking water. Members of Community Against Pollution, a local environmental group, claim that NFC, which makes nuclear fuel bundles and core sub-assemblies, ignores safety regulations and keeps inadequate records of the workers' exposure to radiation and chemicals.

Radhakrishna Rao

Take your partners . . .

Washington

POLITICAL support for the Superconducting Super Collider (SSC), the planned 50-mile circumference proton-proton collider, is becoming harder to sustain as international partners fail to materialize and projected costs climb, senior congressmen told Department of Energy (DOE) officials last week.

"We're getting a little nervous up here," said Representative Tom Bevill (Democrat, Alabama), chairman of the House of Representatives appropriations energy and water development subcommittee, which oversees DOE's budget. Noting that India's promise of \$50 million — less than one per cent of the project's total cost — is the only solid foreign commitment to the SSC so far, Bevill reminded acting DOE energy research director James Decker of promises made to Congress in previous years that contributions from Japan, West Germany and Italy were near. Earlier this year, White House science adviser D. Allan Bromley told another congressional committee that "major" announcements involving the Soviet Union and Japan might be only

SSC director Roy Schwitters says he takes the congressional criticism "very seriously". But he believes that the level of concern indicates a "strong desire to see the project happen" rather than an attempt to undercut it. Nevertheless, he finds it "inexplicable" that DOE has not responded more quickly to growing congressional discontent. "Everybody's nervous" over the lack of an international plan, he says, adding that there is "a lot of interest from [foreign] scientists but they can do nothing until the US expresses its formal interest in establishing a relationship". Decker said that although a draft international plan has been completed for

over a year, it must still be approved by several other agencies as well as DOE secretary James Watkins. A DOE spokesman adds that because the plan depends on the specifics of the SSC's cost and configuration, it is in abeyance until DOE can make an estimate of the cost of the project's latest design modifications, a group of changes that includes doubling the energy of the accelerator's injector and increasing the internal diameter of its magnets (see *Nature* 343, 103; 11 January 1990). The process of determining the SSC's new projected cost is expected to be finished by June, but an initial estimate (which would be enough to go ahead with the international plan) could be done by next month.

G. Christopher Anderson

■ See Correspondence, page 188.

GENETIC ENGINEERING

Modified yeast fine for food

London

THE British government is to allow the commercial development of a genetically manipulated strain of bakers' yeast manufactured by the Dutch company Gist-Brocades. This is the first time any country has sanctioned the development of a food product containing a live genetically manipulated organism (GMO). Other European countries, the United States and Japan are also evaluating the yeast's safety.

No genetic material has been added to the yeast from another species, according to Klaus Osinga of Gist-Brocades. The maltose permease and maltase genes from the yeast were combined with new promoters from another strain of the same species, *Saccharomyces cerevisiae*. The genes and promoters were then spliced back into the yeast genome together with short pieces of 'synthetic' DNA. Osinga says that this DNA contains 'stop' codons, to minimize the chance of any hybrid proteins being produced. The end result is a yeast that should take up and digest maltose more efficiently and release carbon dioxide, which makes bread rise, more quickly.

In its evaluation, the Advisory Committee on Novel Foods and Processes (ACNFP) says it decided that the yeast was safe mainly because the genetic manipulation was within a single species. For the same reason, and following the advice of the government's Food Advisory Committee, bread made using the yeast will not have to carry a label indicating genetic manipulation.

Gist-Brocades' application was also considered, and approved, by two other committees, one in the Department of the Environment and the other part of the Health and Safety Executive, which advise the government on the environmental release of GMOs. Some escape of

the yeast from bakeries is assumed to be inevitable.

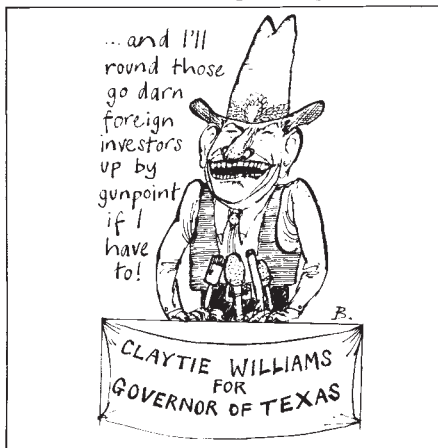
But environmental pressure groups have not welcomed the government's decision. Andrew Lees, of Friends of the Earth, also criticizes the secrecy that surrounded the Gist-Brocades application and which prevented any public debate before the consent was given.

The government's Environmental Protection Bill may soon change these procedures. The bill will disband the two separate committees which advise on the release of GMOs and replace them with a single committee under the chairmanship of Professor John Beringer, of the University of Bristol. Beringer hopes that this committee will not operate with "traditional British secrecy", but says that exact provisions for public access are not yet clear.

Environment minister David Trippier has said that the government is "committed to allowing access to information". But the stage during an application at which information is to be released, and the extent of this information, will be decided in only subsequently. Companies may be permitted to withhold information from the public on the grounds of commercial confidentiality.

John Boodle, of British Fermentation Products (BFP), a subsidiary of Gist-Brocades closely involved with the application, says that Gist-Brocades was concerned about commercial confidentiality because a patent for the yeast has been applied for. But he points out that the company nevertheless chose to submit the yeast to the ACNFP for approval, even though it is not a legal requirement. It was only last November that companies or laboratories intending to release GMOs were required by law to notify the Health and Safety Executive (see *Nature* 341, 681 1989).

Peter Aldhouse



months away. And in testimony last year, DOE mentioned South Korea as a leading candidate for participation.

But Decker said that although DOE was now in the process of putting together an "international plan", the agency has "not moved forward in an aggressive way" on soliciting partners because of uncertainties in the project's cost and configuration.

"I fear congressional support may be eroding on the SSC", said Bill Chappell (Democrat, Florida), a long-time supporter of the project. Congressmen are concerned, he added, that six years after design work on the SSC began, there is still no permanent DOE official in charge of the project. Both Decker and SSC office director Louis Ianiello are holding offices in an acting capacity until permanent officials are named. DOE is currently "considering candidates", for Ianiello's position, says an agency spokesman.

Gupta takes to the hills

London

THE Panjab University at Chandigarh, India, has defended itself against accusations of inertia in investigating the case against Professor V. J. Gupta of the university's Centre of Advanced Study in Geology. In the same document, a press release signed by Ghansham Das, secretary to the vice chancellor, the university announces plans for a field expedition, to be led by Gupta, to sites in the Himalayas where Gupta says he discovered particular fossils, but which his critics declare were acquired elsewhere.

The charges laid against Gupta, which also include the 'recycling' of fossils in different scientific papers and publishing misleading information about the location of fossil sites, were first aired in print in 1988 by Dr John Talent, of Macquarie University, Australia, and four colleagues. Subsequent exchanges between Gupta and Talent appeared in *Nature*, most recently in the issues of 25 January and 1 February of this year (343, 307–308 and 405–406).

Panjab University points out that the authorities became aware of the controversy only in April 1989, whereupon letters were sent to the heads of seven distin-

guished scientific organizations in India informing them of the dispute and of Gupta's willingness to cooperate with any inquiry. The university says that it was interested "not in brushing the controversy under the carpet, but in arriving at the truth". An investigation by two nominees of the Indian National Science Academy had no clear outcome, the report concluding that the authenticity of the fossils concerned would have to be ascertained in the field.

It is proposed that the expedition will consist of seven members — who as yet have not been named — and that it will take place in a few months' time. The stated reasons for the delay in implementing an investigation in the field are that in the Himalayas field work is impossible except in summer, and that it was necessary to find financial support.

The university says that "it is fully aware of its responsibilities, but also aware that it has to work within the limitations of fairness and law". But news that an expedition is planned is unlikely to satisfy Gupta's critics; Talent has stressed that in his view the provenance of the fossils in question can be established unequivocally only by trace-element or isotopic analysis. □

SCIENTIFIC ETHICS

Accusations of "paper recycling"

New Delhi

A NEWLY uncovered case of scientific plagiarism, investigated by India's Society for Scientific Values (SSV), is giving rise to fears that the image of science in India is being tarnished. The victim of this latest fraud, an Indian living in Canada, is concerned that despite the SSV's efforts, local authorities are not taking the stern and prompt actions needed to dispel suspicion and reassure scientists elsewhere that Indian science is to be trusted.

The plagiarism came to light two years ago when O. P. Chandra and his colleague R. M. Barron, fluid dynamicists at the University of Windsor, Ontario, were asked to act as external examiners of a doctoral thesis from the Benaras Hindu University in Varanasi.

Sections of the thesis, they discovered, were identical to papers that they themselves had written in the 1970s. Further investigation established that eight faculty members at BHU had 'recycled' work by Chandra and Barron, publishing the papers in a number of international journals. Chandra and Barron notified SSV and the journals concerned, most of which have now published notices alerting their readers to the fraud. But apart from receiving an assurance from BHU that the charges were being "looked into", Chandra says he is not aware of any action that has

been taken against S. N. Singh and his colleagues at BHU's Department of Mathematics.

Dr A. S. Paintal, president of SSV and director-general of the Indian Council of Medical Research, said that a spate of recent revelations is sully India's image. An Indian geologist has been accused of misrepresenting Himalayan fossils (see *Nature* 343, 405; 1 February 1990), and a zoologist allegedly deceived US and British colleagues by passing off a common English crab as a unique specimen, also from the Himalayas (see *Nature* 342, 333; 1989). SSV, an independent body dedicated to the maintenance of scientific standards, received copies of the plagiarized material from Chandra and Barron, and four months ago communicated its findings to BHU. But the society has no formal powers, and Paintal says that BHU seems to have no intention of punishing the plagiarizers.

According to Paintal, BHU "dismissed a postgraduate student and demoted one scientist, but did nothing to the group leader of the team accused of plagiarism". Chandra's letters to the vice-chancellor of BHU now go unanswered, and he says that editors of *Journal of Mathematical Physics*, one of the journals that inadvertently published copied material, have had a similar lack of response. K. S. Jayaraman

New Zealand shifts ground

Sydney

AUSTRALIA's decision in May last year to campaign for world park status for the Antarctic was mocked by David Lange, then New Zealand's Prime Minister, as a conversion that put Saul's experience on the road to Damascus "in the shade". But ten months later, with the Australian Labor government getting mileage out of its pro-environmental stand as it approaches elections on 24 March, Geoffrey Palmer, now Prime Minister and Environment Minister in New Zealand, has also seen the light.

Facing an election in October this year, he has backed down on New Zealand's original support for a supplementary convention to the Antarctic Treaty that would allow limited mineral exploration.

The Convention on Regulation of Antarctic Minerals Resource Activities (CRAMRA), which would have permitted controlled mining, fell apart last year because both Australia and France refused to ratify it. CRAMRA was initiated by New Zealand, which had always previously sided with the United States in pushing for limited mineral exploration in Antarctica. The present Antarctic Treaty includes no specific provisions on mining.

Despite the many years of work that went into drafting CRAMRA, agreement on the kind of mining convention that should be attached to the treaty is still distant. France, Belgium, India, Australia and Italy have refused to sign CRAMRA and, according to Michael Crawford, spokesman for the Australian Department of Environment, there are indications that the Soviet Union, Finland and Sweden are "swinging towards not signing, with the Federal Republic of Germany in the middle ground". Although the United States administration supports CRAMRA, there is a resolution in the Senate and two bills in the House of Representatives "sympathetic to the Australian stand of a world park", according to Arani Cuthbert of Greenpeace. Meanwhile Chile, Japan, Norway and South Korea remain hard-liners in support of the existing treaty.

New Zealand's change of heart does not, according to Palmer, mean that it has abandoned support for CRAMRA. He says only that New Zealand has "set aside for the moment the question of ratification". Environmental groups are uneasy that New Zealand may gain political points for what they claim is a soft approach. According to Cuthbert, there will be no mining in the next ten years, with or without CRAMRA, because the technology is not ready, so "New Zealand can afford to pretend that mining won't happen".

Tania Ewing

World institute for astronomy

SIR—We would like to offer some additional thoughts on a recent proposal for a world institute for astronomy¹. As a consequence of President George Bush's speech in July 1989, the creation of a permanent scientific base on the Moon is being considered. The lunar surface is one of the best possible sites for an astronomical observatory operating across the entire electromagnetic spectrum, and indeed among the prospects being considered by NASA (the National Aeronautics and Space Administration) are a large (16-metre) optical telescope and an optical interferometer².

It is generally accepted that optical interferometry will be a tool of great astronomical utility in the next century. Several significant Earth-based projects are under way: the European Very Large Telescope, the Optical Very Large Array and a number of ambitious proposals in the United States. Space platforms are also being considered. But in the long term, the lunar surface offers a number of substantial advantages — no atmospheric hindrance at any wavelength, mechanical stability, kilometre-length baselines and an accurately known position.

The establishment of a lunar base, requiring financial and technical resources and complex management, will undoubtedly require an international effort. We suggest that in parallel with this effort, a lunar optical interferometer should be the first astronomical instrument conceived and constructed through worldwide co-operation. The creativity of individual scientists, a necessity for scientific progress, could be stimulated by such a programme, especially in developing countries where a great deal of talent remains unrevealed (*"Ces Mozarts qu'on assassine"*) because of the lack of opportunities and stimulus.

More symbolically, an international scientific venture like this, visible from everywhere on the Earth, would demonstrate a worldwide unity in our quest to understand the Universe. It might be the twenty-first-century equivalent of the rapid dissemination of Galileo's refracting telescope throughout Europe after the discoveries of 1610.

Such a venture would not necessarily demand a world institute of the type proposed by Bahcall *et al.*¹. A delocalized effort, facilitated by rapid communication links and electronic mail, could also make possible a truly worldwide, goal-orientated programme. This 'institute without walls' would naturally deal with the space agencies handling the lunar base itself.

Ground-based interferometers of the type now being developed through individual efforts around the world might also be supported initially by the world institute. If these ideas meet with interest in

the scientific community, the time may be appropriate to hold joint meetings on the 'Lunar Optical Infrared Synthesis Array', and on the possibility of worldwide co-operations towards its construction.

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1. Bahcall, J. N., Giacconi, R., Oda, M., Rees, M., Sagdeev, R. & Sunyev, R. *Nature* **339**, 574 (1989).
2. "Lunar Optical and Infra-red Synthesis Array", workshop proc., Albuquerque, 1989 (in press).

SSC poor value

SIR—The US Secretary of Energy, James Watkins, has convened a 'blue ribbon' panel of 15 'eminent' physicists to advise him on the Superconducting Super Collider (SSC), because he feels that he can count on them to support a decision to proceed with the project, despite numerous design problems and an increase in cost to \$7,000 million. He should have convened a panel of 15 eminent poultry farmers, as they would have had the common sense to avoid two classic errors (1) "Don't put all of your eggs in one basket" and (2) "Don't count your chickens before they hatch", particularly if you have put all your eggs in one basket. US high-energy physicists would obviously like to put all of their 'eggs' in the SSC basket, and they are already counting their unhatched Higgs bosons and their airline tickets to Stockholm.

The situation at the SSC is even more precarious than most people imagine because of the sheer size of the project and the elementary laws of probability. The machine will have nearly 10,000 superconducting magnets. If even one of the magnets is not working properly, the machine will not reach its design energy. Thus, even if each individual magnet is 99.99 per cent reliable, the machine as a whole would have a reliability of $(0.9999)^{10,000} = 37$ per cent. If each magnet were only 99 per cent reliable, the reliability of the whole machine would be 2×10^{-44} . Experience with the prototype superconducting magnets suggests that even 99 per cent magnet reliability would be hard to achieve.

The United States has many pressing problems that demand immediate government attention and financial commitment, including an annual budget deficit of \$200,000 million, an AIDS epidemic, 37 million people without medical insurance and thousands of families who are home-

less. There are more urgent needs for \$7,000 million than a 40-TeV particle accelerator and the search for the elusive Higgs boson.

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Soviet science

SIR—*Nature* often publishes material on the shortcomings of the Soviet Union and East European countries in the organization of science¹, including an interesting Commentary article² on how scientists "striving to be active members of the world community" can be helped. It is very important now that Soviet science is having to be self-supporting. Government subsidies generally go to the central institutes of the USSR Academy of Sciences, but most Soviet scientists are in educational and industrial institutes. Coming as they often do from academic institutes (for various reasons including better opportunities for promotion), the leading scientists there are top quality scientists.

If the goodwill exists, many things could be done:

- (1) Talented young scientists could be allowed to compete for grants and post-doctoral and doctoral student positions in Western institutions (they can at present apply only for A. von Humboldt Foundation grants in Germany).
- (2) Research grants of international or other organizations could be allocated to Soviet scientists for cooperation in fundamental and applied investigations. Even small projects for chemical reagents and spares would be valuable.
- (3) Lecture tours abroad could be of great advantage both for visiting Soviet scientists and for their hosts. Special travel grants could be made.
- (4) Many interesting scientific papers are published only in Russian, only part of them being translated, but much later. Leading international journals could accept worthy papers in Russian, organizing translation themselves, their efforts rewarded by novel ideas and techniques. It is appropriate to mention here that Russians made the preliminary investigations leading later to Nobel prize-winning work, namely that of Barnard for heart transplantation (Demikhov) and of Senger and Gilbert for DNA sequencing (Mirzabekov and Sverdlov).

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1. *Nature* **336**, 2 (1988); **341**, 563 (1989); 101 (1990).
2. Fisher, G. W., Grew, P. C. & Yardley, B. *Nature* **342**, 731–732 (1989).

The case for scientific whaling

Fukuzo Nagasaki

Commercial whaling by Japan, the Soviet Union and Iceland has been widely condemned. But until scientific studies of whale populations are carried out, an informed decision about commercial whaling cannot be made.

OUR ignorance about the biology of whales is delaying any informed discussion about the practice of commercial whaling. Many of those who oppose Japan's whaling operations do so for emotional reasons without scientific basis. Preliminary evidence indicates that some whale populations could sustain responsible commercial hunting.

Many people in the West believe that Japan ignores the International Convention of Regulation of Whaling (ICRW), established in 1946, but in fact Japan's position is one of rational management of whale resources, and conforms both to the spirit and letter of the convention.

Japan's recent scientific whaling programme caused a massive controversy. This programme was established to determine the advisability of lifting the International Whaling Commission (IWC)'s moratorium on commercial whaling, due for discussion later in the year. If the commission

does partially lift the moratorium, Japan will harvest only the southern minke whale, a relatively abundant species and the only one for which there are dependable population analyses available. Japanese whalers have no interest in species such as the blue and sperm whales because of their low stock levels. But this is often ignored in media reports — estimates of minke stocks are rarely reported. The 'revelation' was made last year that the blue whale stock is severely depleted in the Antarctic. But this has been known for 20 years, and the implication that Japanese whalers are interested in the blue whale is both irresponsible and outrageous.

Contrary to these and other accusations, Japan's scientific whaling programme is not a disguised commercial operation. The survey for the 1987–88 season cost ¥1.7 thousand million (\$12 million), and was only partially covered by government subsidy (¥350 million) and the sale of meat (¥1.3 thousand million). The remaining ¥50 million

came from public donations. The 1988–89 survey cost about ¥2 thousand million and came in about ¥300 million over budget.

The whole debate hangs on whether it is acceptable to use whales as a resource. The stage was set for confrontation when the United Nation's conference on the

sions of the scientific committee about how to determine catch–stock relationships. But some representatives felt it would be impossible to eliminate uncertainties. Even Japan conceded that the data from the commercial whalers were likely to be biased, particularly as whalers

tend to concentrate on larger animals in areas of high population density.

To dispel the uncertainty, reliable data were needed. The main parameters needed to determine optimal levels of harvesting are the total population size and the rate of population change. Population size is estimated by sighting surveys; clearer understanding of the population dynamics can then be gained from the natural mortality rate and pregnancy rate by age. The Japanese government drew up a research plan to determine age-specific mortality rates, rather than rates by age group, at the request of the anti-whalers, who claim that this type of analysis is

Oxford Scientific Films

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Minke whale, *Balaenoptera acutorostrata*, surfacing in Antarctica.

Human Environment recommended a 10-year moratorium in 1972. Since then, many concerned groups have entered the debate and ethical, cultural, scientific, economic and political issues have been extensively discussed. Here I confine my discussion to the scientific debate, and mainly to Japanese research on whale stocks.

In 1982, the IWC banned all commercial whaling for 10 years, starting in 1986, without consulting its scientific committee. This decision therefore had no scientific basis. The role of the scientific committee was to determine quotas for stocks exploited. The committee submitted to the IWC an estimate of 260,000 for the total abundance of the southern minke, the species being caught by Japan and the Soviet Union in the Antarctic at that time. But the IWC asserted that because there were no reliable data on any whale population it would be irresponsible to set any quotas. However, it undertook to assess all whale stocks by 1990 at the latest.

Japan therefore participated in discus-

needed. The anti-whalers were well aware that the age-specific-rates method requires larger samples. But Japanese researchers were not daunted by the task, and in 1987 drafted a research plan. The Institute of Cetacean Research was established to gather the basic data necessary for rational management of minke stocks, as well as other species such as fin and sei whales, and to collect general information on Antarctic ecosystems.

The sampling was to be carried out by research vessels using the 'billiard' method to ensure representative sampling. Samples of minke were to be taken close to the pack-ice and from offshore migrating schools. The sample size was to be 1,650 over two consecutive seasons. After a two-year break, another two-year study with the same sample size would provide material for comparison.

In the meantime, US researchers announced that at least 10,000 samples are necessary to estimate natural mortality by age. As some of these researchers were the same people who insisted on Japan's

determining age-specific parameters in the first place, it is difficult not to be suspicious of their true intentions. Another claim, frequently heard, is that all the necessary data can be obtained without lethal sampling. Undeniably, useful infor-

of two of the vessels.

The conclusions from this part of the study are: (1) The density and school size of minke increases markedly in waters near the pack-ice. (2) Sexually mature females tend to congregate in the pack-ice area, immature whales in offshore waters and sexually mature males in both areas. (3) Small, immature whales tend to be solitary, whereas larger, mature males and females form schools. (4) The age distribution revealed a noticeable lack of 5–15-year-old animals (see graph).

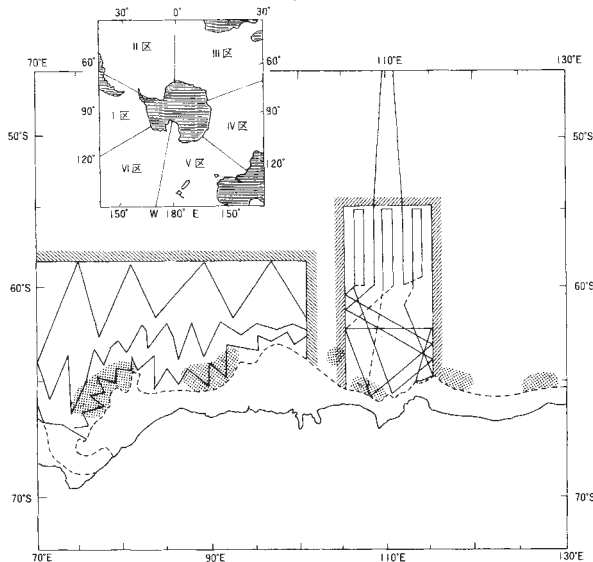
Larger sample sizes are needed to explain this last result. One possible explanation is that the whales are at their most active between the ages of 5 and 15, so could more easily avoid the sampling vessels. Whales at this age were often observed to enter the pack ice, to feed on abundant Krill, where they were beyond the reach of the sampling vessels.

Few data are available on this group and a task of the future will be to determine the age composition of these 'pack ice' whales. Another possibility is that whales of this age are rare because of whaling during the past 10 years. But a sample size of 273 taken during one season is too small to draw any definite conclusions.

Nevertheless, the success of the study does indicate that the same methods of sampling can be applied to a larger area. Indeed, the second fleet sampled an area from south of 52°S to the Ross Sea (77°30'S), between longitudes 168 and 180°E. The sampling was performed from 12 January to 31 March 1989, but the data have not yet been fully analysed. A preliminary analysis, submitted to the IWC in May last year, suggests that: (1) mature and immature whales dominate the northern zones whereas pregnant females are dominant in areas of high whale concentration (such as off Cape Adare); (2) mature and immature whales tend to be solitary, whereas mature females form schools; and (3) there are fewer whales in the waters off Cape Adare, but no distinctive periodical change in biological characteristics emerged. As a result of these two surveys, Japanese researchers are now convinced they can derive an accurate picture of how many minke whales there are by age in a given area. From this information it will be easier to calculate natural mortality by age.

Biological information is not all that is needed for an effective management programme. Whales have a low reproductive rate and live for a long time, and are

thus particularly susceptible to irresponsible exploitation. Japanese and other IWC researchers are developing methods to manage stocks without endangering them. For this, two parameters are used. One is the population level and the other is whether it is moving up or down. If it is known that stocks are increasing, more whales can be caught and vice versa. This adaptive empirical method puts weight not on the total abundance but on the direction of population growth. Some people argue that if the IWC were to approve such a method of management there would be no need to determine age-specific natural mortality. But Soichi Tanaka, a former professor of biomathematics at Tokyo University, believes that a stable catch quota could be fixed sooner if population size and natural mortality rate are known. His opinion is that, if

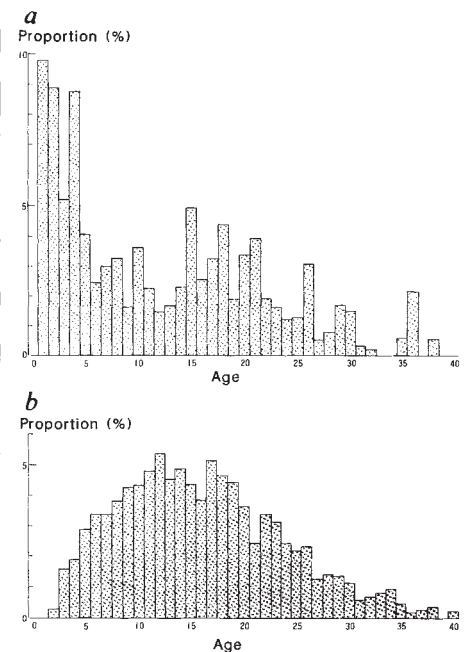


Area covered by research vessels. Main map 1987–88, inset 1984–85. Dotted area, major whaling ground for commercial operations.

mation can be obtained from nonlethal sampling, such as estimates of total abundance. But for assessing population dynamics, it is necessary to kill the animal. In the case of baleen whales such as the minke, the age can be determined by counting the annual rings in the ear plug, for example. Segregative distribution by age, sex and maturity can be determined only by lethal sampling, and this information is essential for the regulation of catches.

Since Japan began its survey, there has been a storm of protest, mainly from the US government, which took the hard-nosed stance that if Japan did not stop all whaling, it would enforce trade sanctions. The Japanese government therefore postponed its plan and instead designed a small-scale feasibility study, which would sample about 300 whales in 1987–88 and the same number again in 1988–89. The first part of the study would be in area IV and the second in area V (see map). All solitary whales and schools of two would be sampled along a particular designated track. In schools of three or more, two whales would be sampled. Some preliminary data would be obtained on the distribution of whales by age and sex.

In the first season (17 January–26 March 1988) three ships covered the area from 55°S to the edge of the pack-ice (about 600 miles) and from 105 to 115°E (about 300 miles; see map). Several species were sighted, and 273 minke (154 males, 119 females) were sampled. The total abundance of minke in this area was estimated to be 7,913 and 9,556 by each



Comparison of the age composition of whales obtained by the 1987–88 survey (a) and by commercial operatives (b).

this procedure is applied, commercial operations could resume as soon as from this year.

Now that the two-year feasibility study is completed, the Japanese government is proceeding with the original 1987 plan. On 12 November last year a small fleet left Japan with the intention of covering the whole of area IV. The sample size will be much smaller than that originally proposed, however, because the Cetacean Research Institute has only limited resources. It is hoped that sample size will be increased in future research. The fleet is expected home in mid April, after which time plans will be reviewed. □

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Have good manners gone for good?

The tendency towards immodesty by the authors of research reports, usually excused as an unavoidable consequence of the competition for research grants, sets a poor example to the young.

LEGEND has it that the seeming civility of academic discourse conceals the longest knives. The late Sir Edward Boyle, who spent much of his adult life in the British House of Commons before becoming Vice-Chancellor of the University of Leeds, used to remark on the difference between his two workplaces. His political opponents, he would say, would disagree forthrightly by accusing him of "talking through his hat", but academic dissenters would instead use forms of words such as "If I might put what the vice-chancellor has just so wisely said in slightly different terms?"

The result, of course, is a certain obliquity of meaning. That convention is still kept. People who disagree with each other in published papers rarely do so in terms so explicit that they cannot be misunderstood. The reasons are understandable enough. So much pride and personal reputation is invested in each research publication that critics sense that explicit criticism would be deeply wounding — and might also be reciprocated. So politeness supervenes.

But polite conventions are not followed universally. On the contrary, one of the most disconcerting features of the research community's present way of working is what seems to be the steady erosion of polite forms of expression.

Trivially, but most commonly, there is a tendency for authors to provide their readers with flattering appraisals of their own work. It seems to have become commonplace that a piece of published work will be described as "the first" of its kind. Great ingenuity is spent on the design of appropriate qualifiers that will legitimize the use of this crucial word.

Plainly it would no longer make sense to say that "we have crystallized copper sulphate for the first time", but even now there must be circumstances in which the repetition of this standard procedure is distinguished from others, whence phrases such as "... for the first time at an elevation greater than 5,000 m". Safeguarding phrases such as "To our knowledge..." are prudently often used.

The impersonal language of the standard communication provides other means for the award of self-credit. Thus one learns to beware of phrases such as "Bloggs *et al.* were the first to show..." and "in the elegant work of Bloggs *et al.*..."; these days, sadly, one should check that Bloggs himself is not among the authors of the

report in which the commendation appears.

But even apparently neutral self-references can be embarrassing, as when Bloggs, weighing the relative merits of alternative hypotheses in the discussion section of a paper, utters "Bloggs *et al.*, on the other hand, hold that...". The impersonality of the third person does not conceal Bloggs's unwillingness to accept that it is for others, not him, to decide which view is correct.

Self-accreditation on these models is damaging not merely because it offends readers, but because, especially when practised by senior people, it is an infectious habit. And while most researchers' struggles for acknowledgement mellow with age and attainment, there seems always to persist a hard core of distinguished people whose past attainments in no way abate their eagerness that their next discovery should be acknowledged as important. It is a poor example to set.

Other routes to immodesty are easily devised, especially in those sections of most research reports describing the antecedents of a new piece of work, and where a false attribution of priority may easily be insinuated. But here is a precis of an item submitted to this journal's Scientific Correspondence section so bowdlerized that (it is hoped) even the author (call him "A") will not recognize it.

"The crystallization of copper sulphate is an important problem. We recently showed that, contrary to the assertion of C in 1985, copper sulphate crystals are Our findings are supported by those of B (published last year in your journal), but we disagree with B in our estimation of (say) the D/H ratio of the water of crystallization. We believe B's estimate is incorrect for the following reasons..."

On the face of things, there is nothing immodest in this simple statement. But in reality, the complainant, A, is the author of an article that has only just appeared in print elsewhere, a good six months after B's article. If anyone deserves the credit for showing that C's five-year-old assertion was incorrect, it is B, not A. The disagreement between A and B, while substantial, is minor compared with the demonstration by both that C was wrong, and appears to be an issue that cannot be settled by argument, but only by further experiment. At their most exalted, A's motives can have been no higher than to grab B's credit for showing that C was

wrong while rubbishing the quality of his work.

Why do people do these things? The standard explanation is that research is now so much more competitive than ever that people must be forgiven for doing everything they can to ensure their own survival. But really? Here is another example from the fast moving field of copper sulphate crystallization. A student, call him K, has a recipe for making crystals on a moving platform and proudly tells the doyen of copper sulphate crystallization at another university, who promptly tells his colleague M.

M tries out the recipe, finds that it works, crystallizes copper sulphate on the upper floor of a moving London omnibus — and sends off a paper for publication. K complains, first to M and then to the editor of the journal. M declines to have K as a co-author because he works in a different discipline, but insists that publication cannot be held up while K follows the traditional path of writing a thesis and then publishing the juicy bits. M's manuscript naturally acknowledges the "assistance generously provided by K".

What seems not fully to be appreciated is that the damage done by affairs like this extends beyond the immediate circle of those involved. K, the student, will no doubt be more careful with information in the future, to the detriment of all those working in the field of copper sulphate crystallization. M will have his publication, and his grant may as a consequence be renewed, but embittered K will be gossiping about his ill-treatment, which in this narrow field will quickly take the gilt off M's gingerbread.

But these, of course, are relatively minor transgressions of the rule that politeness pays. One hears of, and sometimes witnesses, occasions when titans of research clash openly at a scientific meeting. There is, for example, the occasion when X, a practitioner in making copper sulphate crystallize from an aqueous solution of acetic acid, told of his innovation at an international meeting at which the language was foreign to him. The native-speaking chairman, Y, heard him out and then reeled off a string of technical objections whose effect was to persuade the audience that the foreigner must be wrong, and to make the foreigner wish that he had stayed away. The consequences of such incivilities are literally incalculable.

John Maddox

Turbulence and the tube

Katepalli R. Sreenivasan

SIR Horace Lamb, the famed British hydrodynamicist, is said to have remarked, in 1932: "... when I die and go to Heaven, there are two matters on which I hope for enlightenment. One is quantum electrodynamics, and the other is the turbulent motion of fluids. And about the former I am really optimistic." (Ref. 1.) One no longer needs to go to Heaven to seek enlightenment about quantum electrodynamics but, on Earth, turbulence still defies satisfactory description. However, new analytical, experimental and computational tools are being forged today in powerful combination to revitalize efforts at understanding the phenomenon. The report by She, Jackson and Orszag² on page 226 of this issue illustrates the type of information that one can now obtain from careful computational work.

The primary traits of turbulence are that it is vortical (see box) and three-dimensional, and that it manifests itself on many scales. In the familiar example of the Earth's atmosphere, the scale-range (conveniently characterized by the flow Reynolds number) extends from several kilometres to a fraction of a millimetre. The generation and interaction of turbulence on these scales is at the heart of the overall problem, but a full quantitative measurement of small-scale details is at present impossible. Most studies consist of ingenious arguments based on features of the governing differential equations³, temporal measurements from one or at most a few point-probes⁴ and qualitative visualizations⁵. On the other hand, recent computer solutions of the equations of motion have shed light on three-dimensional structures, at least on their kinematic aspects. Although the Reynolds numbers of the most ambitious calculations are still moderate and less than those found in practice — and there remain some finer questions concerning initial conditions, external forcing and the like — computational studies capture

important features of turbulence and their role is firmly established.

She *et al.*² deal with small-scale structure. It has been known for some time that the behaviour on small scales is intermittent and does not follow gaussian statistics. It is believed that large scales do not directly affect small-scale features — at any rate, to a good first approximation for the velocity field — but that small

tube by subjecting it to extension in one direction and compression in the other two; to produce a sheet, one needs extensions in two directions and compression in the third. Kerr^{6,7} pointed out that, on the basis of his calculations, most often two of the principal rates of strain are positive and the third negative, so that the formation of sheet-like structures would be favoured. But, his results do permit a less frequent formation of tubes. Further, as She *et al.* point out, the mere existence of conditions favouring sheet-like structures is not sufficient for expecting them always to occur, because the strain field is not



The small-scale structure of the scalar in a round jet of water at a nozzle Reynolds number of 4,000. From the concentration field of a dye obtained by laser-induced fluorescence (for details, see ref. 9), one constructs a two-dimensional graph of the square of the gradient of the concentration. This quantity represents the small-scale features of the scalar. The resolution of measurement is between one and two Kolmogorov scales. Much finer scales of the scalar have not been resolved.

scales do directly influence the life, size and strength of large scales. If small scales can be modelled satisfactorily, computer simulation of high-Reynolds-number flows of practical interest becomes a viable prospect.

The first contribution of She *et al.* is the demonstration that the high-vorticity-bearing elements are tube-like rather than sheet-like or blob-like. They comment that the structures at lower intensities are sheet-like. A blob can be deformed into a

constant for long enough periods of time over spatial extents commensurate with the observed structures. On balance, it appears that intense tube-like structures coexist with less intense sheet-like structures. Unfortunately, it is not yet clear how one might use this fact in building working models. Nor is the dynamical role of tubes clear. Betchov³ showed that vorticity production requires a preponderance of sheet-like structures. Do we conclude that a large part of vorticity production is associated with low-intensity structures, perhaps because they are more space-filling?

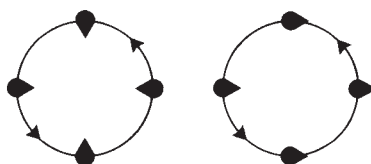
The second main point of the report² is the finding that vorticity and the velocity are roughly aligned, such that the effective nonlinearity is quite small. Basically, from the relation $\mathbf{u} \cdot \nabla \mathbf{u} = \boldsymbol{\omega} \times \mathbf{u} + \nabla(u^2/2)$, it follows that the effective nonlinearity would diminish if $\mathbf{u}$ and $\boldsymbol{\omega}$ were aligned with respect to each other. This conclusion on the depletion of nonlinearity is intriguing and is invoked by the authors to explain the relatively long life of the structures.

It would be instructive to reproduce

THE vorticity, $\boldsymbol{\omega}$, of a fluid motion is related to the velocity field, $\mathbf{u}$, by

$$\boldsymbol{\omega} = \nabla \times \mathbf{u}$$

Despite the apparent simplicity of this mathematical description, the physical meaning of vorticity is less easily described. In essence, non-zero vorticity corresponds to the local rotation of the fluid; more specifically, it means that a volume element of the fluid is changing its orientation in space. Consider the two examples here in which a volume element follows a circular path.



In both cases there is rotary motion of the fluid, but only in the left-hand example is there a change in the spatial orientation of the fluid element, and thus non-zero vorticity. (Note that, more accurately, the element on the right-hand side must also be deformed to maintain zero vorticity.) □

these findings in a laboratory experiment; unfortunately, it will not be possible in the near future. One way of tying together the experimental and computational approaches is to explore areas where their strengths overlap. For example, it is now possible to capture experimentally the three-dimensional field of a passive scalar (such as a small amount of dye or heat) mixed by turbulence. The passive scalar also tends to form intermittent structures by, one might think, the stretching due to the small scale. As She *et al.* demonstrate, this activity is intermittent; so is the small-scale scalar structure. There is experimental evidence to show that the small scales in velocity and scalar fields are poorly correlated spatially. Two uncorrelated intermittent activities may have little to do with each other, so that, even at high Reynolds numbers, small-scale scalar structure may be significantly deformed by the large-scale motion — especially in the case of flows with mean shear. If this is true, it may explain a number of non-universal features observed of small-scale scalar fields. Further, because the large-scale motion is well correlated over sizeable spatial extent as well as over long

enough times, the tendency for small-scale scalar might be to form sheet-like structures.

Some support for this view comes from the figure, where the small-scale structure of the scalar is shown in a section of a turbulent jet. From a cursory study of several parallel sections of the jet, it has been concluded that the structure is indeed sheet-like; this appears to be the case with other inhomogeneous shear flows such as wakes. One can further speculate, on the basis of some experimental results⁸, that the conclusion might also hold for homogeneous shear flows. □

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1. Goldstein, S. A. *Rev. Fluid Mech.* **1**, 1–28 (1969).
2. She, Z.-S., Jackson, E. & Orszag, S. A. *Nature* **344**, 226–228 (1990).
3. Betchov, R. J. *Fluid Mech.* **1**, 497–504 (1956).
4. Corrsin, S. & Kistler, A. L. *NACA Tech. Rep.* 3133 (1955).
5. Schwarz, K. W. *Phys. Rev. Lett.* **64**, 415–418 (1990).
6. Kerr, R. M. *J. Fluid Mech.* **153**, 31–58 (1985).
7. Kerr, R. M. *Phys. Rev. Lett.* **59**, 783–786 (1980).
8. Sreenivasan, K. R. & Tavalouris, S. J. *Fluid Mech.* **101**, 783–795 (1980).
9. Prasad, R. R. & Sreenivasan, K. R. *Exp. Fluids* **7**, 259–264 (1989).

GENE EXPRESSION

Chromatin contract to silence

Bruce Alberts and Rolf Sternglanz

In female mammals, the chromatin in one of the two X chromosomes remains condensed in a mitotic-like conformation throughout interphase and is largely non-transcribed. This type of compact, inactive heterochromatin is also associated with position-effect variegation in the fruit fly, *Drosophila melanogaster*^{1,2}, in which it is responsible for an inherited form of gene inactivation. On page 219 of this issue, Reuter *et al.*³ report the use of a combination of genetics and molecular biology to identify a protein in *Drosophila* that seems to be intimately involved with this type of heterochromatin formation.

This protein is the product of the *Suvar(3)7* gene at chromosome position 87E. Reducing the number of copies of this gene from two to one suppresses the extent of position-effect variegation (that is, it decreases the amount of heterochromatin), whereas increasing the number of copies to three or more enhances variegation progressively in response to gene dose. Genes having this property could well encode structural components of heterochromatin. Indeed, a second gene that also increases the extent of position-effect variegation in proportion to gene dose, *Suvar(2)5* (also known as *Su(var)205*), is known to encode a *Drosophila* protein that is confined to heterochromatin, called HP-1 (ref. 4; S. Elgin, personal communication).

What makes the proteins encoded by *Suvar(3)7* and *Suvar(2)5* particularly interesting is the unusual nature of the gene regulatory processes that they affect. When a chromosome rearrangement joins the middle of a region of heterochromatin to a region of the chromosome that is normally packaged into standard chromatin, a zone of gene inactivation spreads progressively from the chromosome breakpoint to cover one or more adjacent genes. Most strikingly, although the extent of this heterochromatin spreading is different in different cells of the embryo, the inactivated zone is stably inherited by the progeny of each of these cells. Thus, in position-effect variegation, as in mammalian X-chromosome inactivation, genes are turned off in a manner that seems to be inherited from parental chromosome to daughter chromosomes.

The inheritance of the repressed gene state cannot be attributed to feedback loops of diffusible gene regulatory proteins, because the second copy of each affected gene in a diploid cell remains normally active. The models proposed to explain this form of gene regulation instead suggest that heterochromatin forms by a highly cooperative, 'crystallization' event, which is nucleated from special chromosomal sites and then spreads along the chromosome to take in hundreds of kilobases of DNA⁵. It seems

that the ability to create new regions of heterochromatin is lost early in embryogenesis and that, thereafter, the established pattern of heterochromatin is inherited. One might propose that some proteins in the heterochromatin remain bound to the DNA when it is replicated, being inherited by the two daughter DNA helices along with the histone octamers from the parental nucleosomes⁶. These proteins would then nucleate the cooperative reassembly of heterochromatin on each daughter chromosome.

The orderly development of multicellular organisms requires that progeny cells 'remember' the patterns of gene expression of their parents. To what extent is this cell memory dependent on chromatin-bound proteins that are directly inherited during DNA replication, as postulated for the maintenance of heterochromatin?

Models of this type are generally not invoked to explain the regulation of individual genes. But a popular model for the regulation of the bithorax complex of homeotic genes invokes the progressive inactivation of large chromosomal regions⁷, and there are reasons to believe that genes in the *Polycomb* class might act to keep genes turned off in the bithorax complex by mechanisms analogous to those in heterochromatin formation¹.

Help in analysing the effect of chromatin structure on the expression of genes should also come from recent work on the yeast *Saccharomyces cerevisiae*. Yeast chromosome III has three widely separated genetic loci that code for mating-type information. Only one of the loci, *MAT*, is expressed; it determines the mating type of the cell, *a* or *α* haploid, or *a/α* diploid. The other two loci, *HML* and *HMR*, are transcriptionally silent, in spite of the fact that they contain the same DNA sequences (*a* or *α*) as the expressed locus, *MAT*. If the mating type genes *HML* or *HMR* are replaced by other yeast genes, these will also be kept transcriptionally repressed. The repression is through *cis*-acting DNA sequences, termed silencers⁸, which are separated by a kilobase or more from the promoters of the genes whose expression is affected. The silencers contain autonomously replicating sequence elements that can function as origins of replication; DNA replication has in fact been implicated in the establishment of silencing⁹.

The silencers also have binding sites for two abundant DNA-binding proteins, RAP1 (ref. 10) and ABF1 (ref. 11). These two proteins bind upstream of many important housekeeping genes, and they are essential for the viability of the yeast cell. Genetic analysis has shown that four additional proteins, SIR1–4, are necessary for silencing¹². The genes encoding the four proteins have been cloned, sequenced and mutated, but their functions are still unclear. Interestingly,

histone H4 is important in silencing: deletions within the evolutionarily conserved amino-terminal sequence do not interfere with cell viability, but silencing is severely affected¹³. The positively charged amino acids at positions 16–18 of H4 have been found to be crucial for silencing¹⁴, and there is evidence that the SIR3 protein interacts with this region of the histone (L. Johnson and M. Grunstein, personal communication). This and other evidence suggests that an altered chromatin structure in the region of the *HML* and *HMR* loci is responsible for the transcriptional silencing.

The Suvar(3)7 protein has no obvious sequence similarity to the other known *Drosophila* heterochromatin protein, HP-1 (ref. 15), and neither of these *Drosophila* proteins is similar in sequence to any of the yeast proteins implicated in silencing. Although the relation between yeast silencing and *Drosophila* heterochromatin is still speculative, novel interactions between non-histone and histone proteins seem to be involved in both systems.

The genetics indicate that there could be up to 6–10 *Drosophila* genes that share the properties of Suvar(3)7 in being both a haplo-abnormal suppressor and a triplo-abnormal enhancer of heterochromatin formation¹⁶. Do the proteins that they encode bind to nucleosomes and/or to each other, as predicted by the cooperative assembly model? So far, the genetics and gene cloning are far ahead of the biochemistry in these systems. But researchers throughout the world are thawing out their old preparations of histones, and it seems that we are about to experience a major rebirth of interest in the structure of chromatin. □

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When two's company and three's a correlation

Keith Codling

NATURE has been surprisingly kind in allowing us to understand the structure of complex atoms (and, indeed, molecules) in terms of a rather simple model in which each electron moves independently of each of the others. This works because the interaction between any pair of electrons can usually be neglected when compared with the interaction of each electron with the atom as a whole. Nevertheless, this simplicity has encouraged spectroscopists to explore what happens when a few electrons are elevated above the crowd, in 'planetary atoms', so that their pairwise interactions become important. A new study of doubly excited barium (Ba^{++} , e^- , e^-) by Eichmann *et al.*¹ shows just how strong the correlations between such planetary electrons can become.

In the standard independent-particle model of atoms, each individual electron is regarded as moving in the combined fields of the positive nucleus and some average distribution of all the other electrons. The state of each electron can then be well defined by its ' nl ' value, where n indicates its (fixed) energy and orbital size, and l its precise angular momentum. Increasing the angular momentum means that an electron penetrates less and less into the core of the atom. Atomic spectroscopy is largely a matter of finding the details of states available to electrons through their interaction with electromagnetic radiation.

When two electrons are promoted well outside the valence shell of an atom, their motion adjusts to the combined influence of their mutual repulsion and the net attraction of the residual core. One important motivation for studying such electron correlation effects is that analogous correlations must surely underlie the course of chemical and biochemical transformations, whose detailed physical

mechanisms are the subject of vigorous research.

The single-photon absorption spectrum of helium obtained by Madden and I^{2,3} in 1963 yielded the first experimental evidence of the complete breakdown of the independent-particle model. The soft X-rays used, which had sufficient energy to remove one electron entirely from the atom, instead promoted both the electrons. Had they remained independent, the spectrum should have comprised two series of spectral lines with equal strength, but what we saw (Fig. 1) was one strong series (labelled +) and one very much weaker one (–).

A new classification scheme was required to describe the angular and radial correlations between the two electrons. This is clearly the archetypal three-body problem, with two excited electrons and a bare, doubly charged helium nucleus, so that it has been the subject of much theoretical effort^{4,5}.

Since then, the use of 'multiphoton' excitation has allowed a more general attack on the atomic three-body problem. It provides access to states forbidden to us in single photon excitation and a far superior resolution. Recent work^{6,7} on the doubly excited states of the alkaline earth elements calcium and barium has been hampered by the penetration of the outer electrons into the core of residual electrons surrounding the nucleus: the central third body was poorly defined.

Eichmann *et al.*¹ of the University of Freiburg overcame this problem by creating planetary atoms in which the inner of the two excited electrons was promoted into states with high angular momentum, so truly avoiding the core. The creation of such atoms was achieved by sequential resonant excitation of barium atoms using six lasers (Fig. 2). Energetically this posed no problem because all transitions involved laser photons with energies of about 2.5 electron volts (visible region), but technically it was quite an achievement. Two excimer-pumped dye lasers excite the first valence 6s electron to states with $n > 60$ and $l = 2$ via a single intermediate state. A set of three more dye lasers excites the second electron via two intermediate states. Fortunately, the first excited electron remains an uninvolved 'spectator' of the excitation of the second electron. The final transition of the second 6s electron to planetary states ($n > 30$, $l = 2$ or 4) lying just below the Ba^{++} ionization threshold is driven by the sixth dye laser.

The experimental spectrum of Ba^{++} counts, as a function of the energy of the

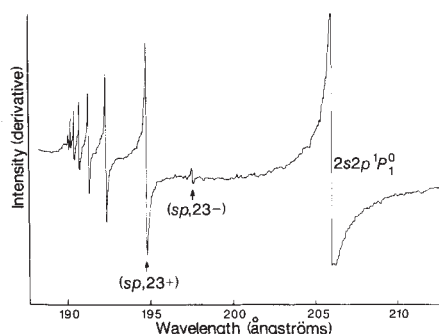


FIG. 1 Soft X-ray absorption spectrum of helium² around a wavelength of 200 ångströms, equivalent to an excitation energy of 60 electron volts, showing two sequences of lines of differing strength.

1. Baker, W.K. *Adv. Genet.* **14**, 133–169 (1968).
2. Spofford, J.B. in *The Genetics and Biology of Drosophila* Vol. 1c (eds Ashburner, M. & Novitski, E.) 955–1018 (Academic, New York, 1976).
3. Reuter, G. *et al. Nature* **344**, 219–223 (1990).
4. Eissenberg, J.C. *Bioessays* **11**, 14–17 (1989).
5. Tartof, K.D., Bishop C., Jones, M., Hobbs, C.A. & Locke, J. *Dev. Genet.* **10**, 162–176 (1989).
6. Bonne-Andrea, C., Wong, M.L. & Alberts, B.M. *Nature* **343**, 719–726 (1990).
7. Peifer, M., Karchi, F. & Bender, W. *Genes Dev.* **1**, 891–898 (1987).
8. Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R. & Nasmyth, K. *Cell* **41**, 41–48 (1985).
9. Miller, A.M. & Nasmyth, K. *Nature* **312**, 247–251 (1984).
10. Shore, D. & Nasmyth, K. *Cell* **51**, 721–732 (1987).
11. Diffley, J.F.X. & Stillman, B. *Science* **246**, 1034–1038 (1989).
12. Rine, J. & Herskowitz, I. *Genetics* **116**, 9–22 (1987).
13. Kayne, P.S. *et al. Cell* **55**, 27–39 (1988).
14. Meggie, P.C., Morgan, B.A. & Smith, M.M. *Science* **247**, 841–845 (1990).
15. James, T.C. & Elgin, S. *Molec. cell. Biol.* **6**, 3862–3872 (1986).
16. Wustmann, G., Szidonya, J., Taubert, H. & Reuter, G. *Molec. Gen. Genet.* **217**, 520–527 (1989).

sixth laser, shows clear evidence of correlation effects. Because the two excited electrons have greatly differing radial extensions, the correlation reduces in this case to a Stark effect of one electron on the other. (Conventionally, the Stark effect is the effect of an external electric field on an atom.) Here, the outermost electron could be imagined to be frozen in space⁷, creating an electric field and inducing a dipole interaction between the outer electron and the polarized, excited Ba^+ core. This interpretation was cleverly checked by removing the outermost electron completely, replacing it by an equivalent external electric field and reproducing almost exactly the planetary component of the Ba^{++} spectrum.

The authors obtained detailed information on the correlated motion of the two electrons. The charge-density plots of the inner electron's wavefunction (Fig. 3a,b) show two extremes of behaviour, when the two (3d and 7d) electrons are predominantly on opposite sides of the Ba^{++} core and when they are on the same side. This constitutes dramatic evidence of the breakdown of the independent particle model. On the other hand a typical core-penetrating state, such as the 3d7d, exhibits much less positional correlation (Fig. 3c), with corresponding independent-particle behaviour. It is interesting to note that there is a direct correspondence between this behaviour in barium and the + and - states of doubly excited helium, which can themselves be

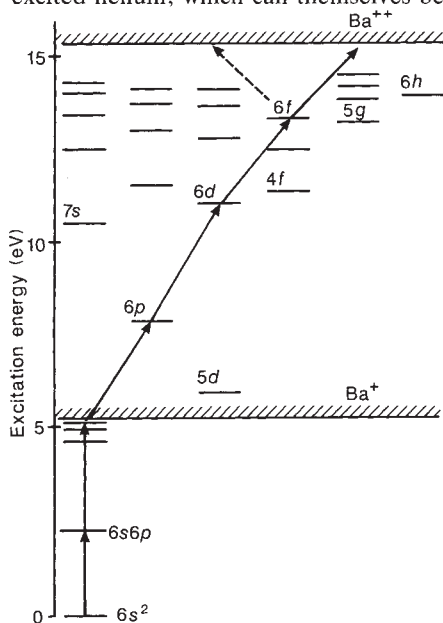


FIG. 2 Six-photon excitation scheme for generating planetary barium atoms. The scheme is resonant because each step ends on a particular state nl of barium. The two electrons start in the valence $6s$ shell, and are then excited separately. The terms $s, p, d, f, g, \dots$ correspond to an increasing number of units (0, 1, 2, 3, 4, ...) of angular momentum, reducing the core penetration. The final step leaves the inner electron in either a d - or a g -state.

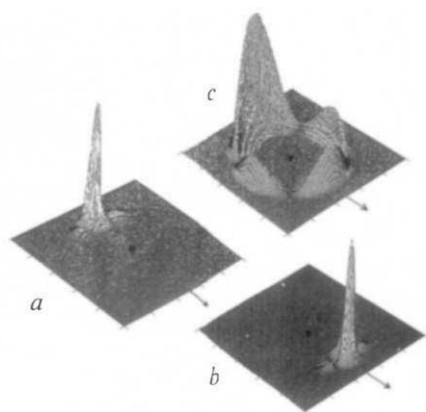


FIG. 3 Charge-density plots for the inner of the two planetary electrons in doubly excited barium. The arrow shows the direction to the outer 7d electron. (From ref. 1.)

imagined to involve a Stark-state-like superposition of states.

One major drawback in the study of the three-body problem has been the lack of enough systematic data of sufficient

TRANSFER RNA

Synthetases gain recognition

Dino Moras

A KEY step in the accurate translation of genetic information is the reaction by which transfer RNAs are charged with the appropriate amino acids. This step is catalysed by enzymes known as aminoacyl-tRNA synthetases, each of which must recognize a particular amino acid and its cognate tRNA. The problem of how these enzymes recognize their tRNA is attracting particular attention at the moment, the hope being that an understanding of the process may shed light on the origin of the genetic code. A major step towards the solution of the problem has now been taken with the determination by Rould and colleagues¹ of the crystal structure of a complex between a glutamyl-tRNA synthetase with the tRNA specific for glutamine ($tRNA^{Gln}$) and ATP, providing the first direct picture of an intermediate in the charging of a tRNA at atomic resolution. Incidentally, the molecular structures of a new tRNA and a new synthetase are revealed, allowing interesting comparison with the few structures known to date.

The aminoacyl-tRNA synthetases form a family of proteins characterized by their high degree of structural diversity; they vary in size and oligomeric state, and there is only limited sequence homology between different enzymes. The crystal structures of two of the enzymes have been determined previously: tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* and methionyl-tRNA synthetase of *Escherichia coli*^{2,3}, both of which form active dimers in solution. Glutamyl-tRNA synthetase is the first

resolution. Clearly, multiphoton excitation provides a route to this objective and planetary atoms constitute a relatively simple but realistic example of a non-separable quantum system with a clear classical counterpart. During the coming years we can expect to discover new collective phenomena in atomic and molecular systems and it is important to understand in detail the simplest non-trivial example of correlations if one is to progress further. □

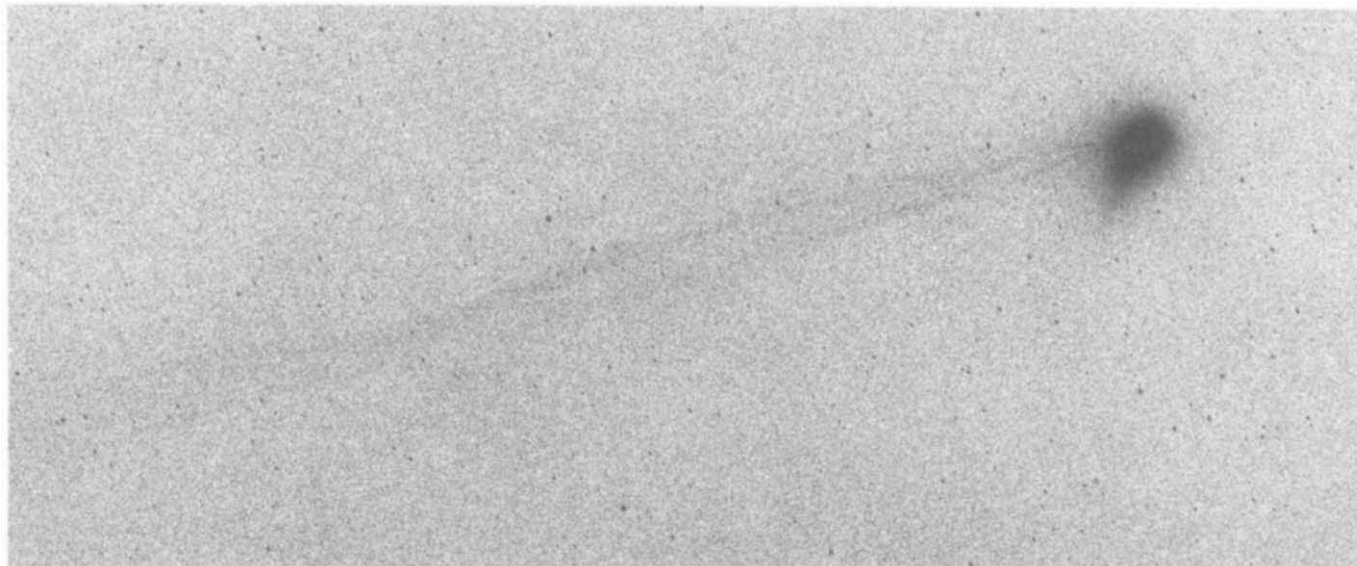
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1. Eichmann, U., Lange, V. & Sande, W. *Phys. Rev. Lett.* **64**, 274–277 (1990).
2. Madden, R.P. & Codling, K. *Phys. Rev. Lett.* **10**, 518–521 (1963).
3. Madden, R.P. & Codling, K. *Astrophys. J.* **141**, 364–375 (1965).
4. Fano, U. *Rep. Prog. Phys.* **46**, 97–165 (1983).
5. Herrick, D.R. *Adv. chem. Phys.* **52**, 1–115 (1983).
6. Morita, N. & Suzuki, T. *J. Phys. B* **21**, L439–L444 (1988).
7. Camus, P. *et al. Phys. Rev. Lett.* **62**, 2365–2368 (1989).

monomeric synthetase to be analysed. The protein has an unusual elongated ellipsoidal shape and is divided into four domains, of which the most important functionally is a five-stranded parallel β -sheet folded in a manner similar to the so-called Rossman fold. This structural motif was first observed in the dehydrogenases and then in many other proteins that bind mono- and dinucleotides. Its occurrence in synthetases was first predicted in 1974 along with the hypothesis of its origination from a primer model gene⁴. This domain is also present in tyrosyl- and methionyl-tRNA synthetases, prompting Rould *et al.* to suggest that all synthetases could have evolved from this common domain, which achieves most of the enzyme's functions. In Gln-tRNA synthetase it is involved both in the recognition process of the acceptor stem and in the formation of the catalytic site. This appealing generalization is not supported, however, by the latest results of the European Molecular Biology Laboratory group at Grenoble, which has solved the crystal structure of *E. coli* Ser-tRNA synthetase: this enzyme does not have a Rossman fold (S. Cusack and R. Leberman, personal communication). Therefore it appears that the synthetase family can be divided into at least two subgroups.

The structure of complexed $tRNA^{Gln}$ has the L-shape characteristic of tRNAs, but when compared with other uncomplexed tRNAs it shows important differences. The most remarkable are the disruption of the first base pair between nucleotides 1 and 72, and the unusual con-

Brighter days ahead for Comet Austin



COMET Austin, now 0.75 astronomical units (AU) from the Sun and 1.5 AU from the Earth, shows two tails in this European Southern Observatory (ESO) photograph (a 6-minute exposure taken on 24 February from La Silla, Chile). The long tail, which extends across more than 2 degrees of the sky, is a trail of ionic material evaporated from the comet's icy surface by the Sun's heat and swept back by the solar wind; the wiggles are caused by solar-wind variations and the interplanetary magnetic field. The second, much shorter tail is a trail of dust particles reflecting sunlight.

Comet Austin will be at its closest to the Sun in April. Its brightness over the coming weeks is unpredictable, depending on the amount of evaporated cometary material —mostly ice and dust — that is available to

form an extended 'coma' of reflecting material. This observation of the tail, along with some recent spectroscopic data, indicate that many species of gaseous ions are beginning to boil off. But the brightness of Comet Austin is presently falling a little behind the most optimistic predictions, which would have made it as bright as any star in the sky near its perihelion (the point of its orbit where it is closest to the Sun). From the Northern Hemisphere, the comet will appear brightest in late April and May, after it has gone round the Sun and is on its way towards Earth.

The predictions of Comet Austin's progress are made more difficult because analysis of its orbit indicates that it is a 'new' comet, approaching the Sun for the first time. The standard theory has it that a

large reservoir of comets, the Oort cloud, orbits the Sun at some ten thousand AU, and that from time to time gravitational perturbations or collisions send new comets on highly elliptical or hyperbolic orbits towards the inner Solar System, where they may or may not be gravitationally captured. Periodic comets, such as Halley, have approached the Sun many times, and their behaviour at each pass is fairly consistent. But a new comet such as Austin is being heated by the Sun for the first time, and its brightness depends on the quantity of volatile materials that can be boiled off its pristine surface to form the visible coma. The current prediction from ESO is that Austin will reach a peak brightness around magnitude zero, brighter than all but a few stars.

David Lindley

formation of the 3' CCA end, which forms a hairpin. There are also variations in the anticodon loop and stem, where the U35 central base of the anticodon is stacked underneath A37, and bases C34 and G36 are unstacked to project outwards. These conformational differences are probably induced and stabilized by interaction with the enzyme, so recognition could well follow an induced fit mechanism. Assuming that the structure of free tRNA^{Gln} is typical of other known tRNAs, this first description of a complexed tRNA provides another demonstration of the flexibility of these adaptor molecules. It would be interesting to analyse the uncomplexed synthetase to see whether comparably large conformational changes occur at the protein level.

Sequences important in the recognition of tRNA have been mapped *in vivo* and *in vitro* in molecular genetic experiments: it turns out that a limited number of nucleotides are responsible for the specific recognition of a tRNA by its cognate synthetase⁵. The significance of a small number of key base pairs at the end of the

acceptor stem is well documented: for example, the role of the pairing between the bases at positions 3 and 70 is emphasized in the *E. coli* alanine system, where any alteration affects specificity⁶. One surprising feature of the molecular interactions between tRNA^{Gln} and its synthetase is that there are a very large number of them. They extend along one side of the L-shaped tRNA in a way that has been suggested by Rich and Schimmel⁷. Three major recognition elements can be identified. First, three polar amino-acid side chains and a buried water molecule interact with the base pairs at positions 3 and 70, and 2 and 71 in the minor groove. The second determinant is the anticodon loop. The third results from the ability of nucleotide G73, the 'discriminator base'⁸, to assume an unusual conformation by hydrogen-bonding to the phosphate of A72 via a hairpin turn. All these recognition elements are sequence-specific. Surface complementarity associated with hydrogen bonding is also likely to be a major discriminating factor.

What about a general recognition code,

the so-called second genetic code? The idea of a simple recognition code arising from direct tRNA interaction with its corresponding amino acid⁹ is discarded by Rould and his collaborators because no such direct interactions are found. They point out that the complexity of the protein-RNA association precludes the concept of a code in any conventional sense. One can speculate that the common determinant (namely, the end of the acceptor stem) might be recognized differently in the various systems. Even in the absence of sequence-specific structures (such as the G73-dependent hairpin loop), there are alternative solutions to the recognition problem. In the Gln-tRNA synthetase complex, recognition of the CCA stem proceeds through the minor groove of the tRNA, but this is not an absolute requirement because the major groove side is also accessible at the end of the double-helical stem. These observations could explain why the aminoacyl-tRNA synthetase family is so heterogeneous and why structural homologies are so limited. The origin of these

proteins and the nature of their evolution remains an open question. The crystal structure of glutamyl-tRNA synthetase complexed with tRNA^{Gln} and ATP has illustrated a key intermediate along the

1. Rould, M.A., Perona, J.J., Söll, D. & Steltz, T.A. *Science* **246**, 1135–1142 (1989).
2. Brick, P., Bhat, T.N. & Blow, D.M. *J. molec. Biol.* **208**, 83–98 (1989).
3. Brunie, S. *et al. J. molec. Graphics* **5**, 18–21 (1987).
4. Rossman, M.G., Moras, D. & Olsen, K.W. *Nature* **250**, 194–199 (1974).
5. Normanly, J. & Abelson, J. A. *Rev. Biochem.* **58**, 1029–1049 (1989).
6. Hou, Y.-M. & Schimmel, P. *Nature* **333**, 140–145 (1988).
7. Rich, A. & Schimmel, P.R. *Nucleic Acids Res.* **4**, 1649–1665 (1977).
8. Crothers, D.M., Seno, T. & Söll, D. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3063–3067 (1972).
9. de Duve, G. *Proc. natn. Acad. Sci. U.S.A.* **69**, 117–118 (1972).

pathway of genetic translation. It opens the door to a fascinating world where genetics and biochemistry can be interpreted in the light of crystallographic results to clarify structure–function relationships. When more structural information comes to hand for different systems, it may be that common features can be derived from the apparent complexity of molecular recognition to establish the structural principles behind tRNA interactions with their synthetases. □

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PARTICLE PHYSICS

Deep resonance of CP violation

John Ellis

*In a way
It was fun
Not to see Mount Fuji
In foggy rain'.*

AN experimental group working at the Institut Laue-Langevin (ILL) in Grenoble has just published² a new upper limit on the electric-dipole moment of the neutron: $|d_n| < 1.2 \times 10^{-26} e \text{ cm}$, where e is the unit of electric charge. Scaled up from the radius of the neutron (10^{-13} cm) to the radius of the Earth, this would correspond to a charge 'mountain' at most $10 \mu\text{m}$ high on the surface of the Earth — less than 10^{-8} the height of Mount Fuji. The interest in the search for an electric-dipole moment is that it would violate the combination of charge conjugation invariance and parity known as CP symmetry. As such, any electric-dipole moment would take the opposite sign for the antineutron, and thus discriminate between matter and antimatter. Some even speculate that it could be related to the global abundance of matter and absence of antimatter in the Universe.

The first experimental limit on the neutron's electric-dipole moment, $|d_n| < 5 \times 10^{-20} e \text{ cm}$, was given³ in 1951, and the upper limit has steadily improved since then by better than two orders of magnitude every decade. The most recent limit before the ILL's, $|d_n| < 2.6 \times 10^{-25} e \text{ cm}$, was provided⁴ by a group working at an experimental nuclear reactor near Leningrad in 1986. Post-Chernobyl prudence closed the reactor for some time, but the group is resuming measurements with an improved apparatus.

The ILL experiment is shown in Fig. 1. Ultra-cold neutrons from a turbine pass through a polarizing foil and are stored in a 'bottle' with reflecting walls inside five layers of mu-metal shielding. The neutrons are subjected to an applied

magnetic field of 1 microtesla and an electric field: the experiment seeks a shift in the spin precession frequency when the electric field is reversed. The neutron spins are allowed to precess around the magnetic field axis for about 70 s before being extracted, then those in the appropriate spin state are extracted through the polarizing foil, used now as a spin analyser. Subsequently, the spins of the neutrons remaining in the bottle are flipped, and then also extracted through the polarizing foil. During the experiment, the magnetic field is monitored by three optically-pumped rubidium magnetometers inside the mu-metal shielding, but outside the neutron bottle.

The data from 15 reactor cycles over three years are shown in Fig. 2. They give $d_n = -1.9 \pm 2.2 \times 10^{-26} e \text{ cm}$. There seem to be systematic effects, such as a correlation of the magnetic field with reversals of the electric field, that could mimic a neutron electric-dipole moment. These can be partially corrected using data from the external rubidium magnetometers to give a best estimate $d_n = -(3 \pm 5) \times 10^{-26} e \text{ cm}$, or $|d_n| < 1.2 \times 10^{-26} e \text{ cm}$ at the 95 per cent confidence level². Future plans include a gas magnetometer inside an enlarged bottle within a four-layer mu-metal shield. This should permit the present upper limit on $|d_n|$ to be improved by about an order of magnitude, insufficient to test the standard model of particle physics, but enough to embarrass many of its extensions.

In this standard model, CP is violated by phases in the couplings to quarks of the charged vector bosons $W^\pm$, mediators of the weak nuclear force. At least four such

couplings, and hence two $W^\pm$ exchanges, are needed to make d_n non-zero. An important ambiguity in calculating d_n has recently been removed by the discovery⁵ at LEP that there are only six quarks, in which case there is only the single CP-violating phase discovered by Kobayashi and Maskawa⁶, whereas there would have been three phases if there had been eight quarks. The 'KM' phase can in principle be fixed by measurements of other CP-violating quantities such as the mass-mixing parameter ε for the K^0 and anti- K^0 mesons and the parameter ε' characterizing $K \rightarrow 2\pi$ decay amplitudes, subject to our continuing ignorance of the mass of the still-unobserved top quark t .

In practice, whereas ε is well measured, there is some discrepancy^{7,8} between the two most precise reported values of ε' , which needs to be resolved before this can be used to constrain any standard model parameters. Moreover, there is still some theoretical uncertainty in the value of ε' expected for any given value of the top mass, though it has recently transpired⁹ that ε' probably vanishes for some value of $m_t \approx 200 \text{ GeV}$. Direct experimental searches^{10,11} tell us that $m_t \geq 80 \text{ GeV}$, whereas indirect arguments¹² based on radiative corrections to electroweak measurements favour $m_t \approx 135 \pm 30 \text{ GeV}$. The uncertainties in m_t and the KM phase limit theorists' ability to calculate d_n in the standard model, and there are also large uncertainties introduced by our inability to calculate in the strong interactions, so the best one can say¹³ in the standard model is that $d_n \approx 10^{-33} - 10^{-31} e \text{ cm}$. This is far below the present experimental sensitivity, and indeed beyond the reach of any improvement currently envisaged. All the more reason

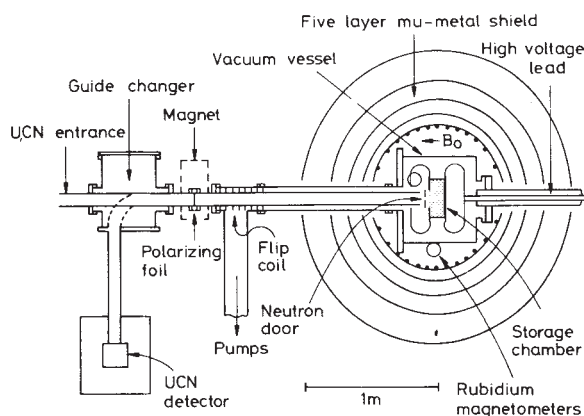


FIG. 1 The Institut Laue-Langevin apparatus for measuring d_n . Ultra-cold neutrons are stored for 70 s in a magnetic field $1 \mu\text{T}$ and an electric field of around 1 MV m^{-1} . (From ref. 2.)

to look, because any non-zero result would be evidence for new physics.

There is one other possible source of CP violation in the standard model, namely a phase θ characterizing non-perturbative strong-interaction effects in quantum chromodynamics, the strong force theory. The ILL result tells us that $\theta \approx 10^{-9}$: why

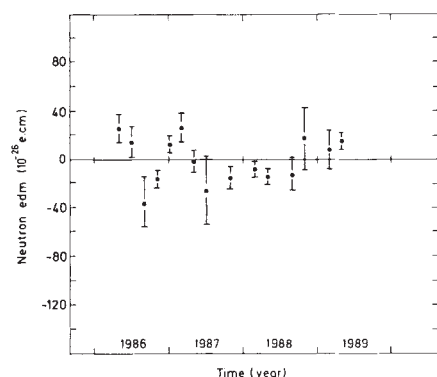


FIG. 2 The values of d_n measured during each reactor cycle, before application of the magnetometer corrections. (From ref. 2.)

should this be so? One suggestion is that the standard model should be extended slightly, introducing an approximate symmetry that guarantees $\theta \approx 0$. The best known such extension¹⁴ on the market introduces a light spin-zero boson, the axion, and suggests¹⁵ that $\theta \approx 10^{-16}$, again far below the present experimental sensitivity. Particle physics, astrophysics and cosmology severely constrain the possible properties of the axion, which is nevertheless a fancied candidate for the dark matter of the Universe and is actively sought in experiments.

The standard model would need to be extended further to obtain d_n comparable to the ILL limit, and one favoured suggestion is 'supersymmetry' — the notion that another set of, as yet unknown, 'sparticles' parallels the known particles. In addition to the KM phase, supersymmetric models contain other possible sources of CP violation¹⁶ which could even give $d_n \gg 10^{-26}$ e cm. In particular, Weinberg¹⁷ has recently identified a set of quantum effects that were previously disregarded, but are likely to make the dominant contributions to d_n in a large class of models including supersymmetry. The supersymmetric CP-violating phases are severely restricted by the ILL measurement, but nowhere near as

stringently as θ in quantum chromodynamics. It is premature to conclude that supersymmetric models are in deep trouble, but supersymmetric modellers should beware, and it would be very interesting to improve the ILL upper limit by another order of magnitude.

One model that does seem to be excluded by the ILL result is that in which the dominant source of CP violation in the K^0 - anti- K^0 system is a complex phase associated with multiple Higgs bosons⁸. This model was already in trouble before the ILL result, and its difficulties have been aggravated by the new quantum effects recently identified.

In view of all these problems raised by the ILL result for extensions of the good old standard model, why bother to extend it all? There is one other apparently CP-violating quantity that seems inexplicable within the standard model, namely the matter-antimatter asymmetry in the Universe. Following the seminal work of Sakharov¹⁹, the consensus has grown that

this could and should be calculable in terms of CP-violating particle interactions. Grand unified theories which are complicated enough to do this²⁰ tend to have a large quantum-chromodynamic θ parameter and hence $d_n \geq 10^{-27}$ e cm, not far below the ILL limit. However, non-perturbative electroweak interactions may wash out any GUT matter-antimatter asymmetry²¹. In principle, they could also regenerate it, but it has not yet been shown how to achieve this with only the minimal standard model and its KM phase. The ILL result on the neutron electric-dipole moment certainly has many ramifications for both particle physics and cosmology:

*Breaking the silence
of an ancient pond
a frog jumped into the water
a deep resonance¹.*

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PROTEIN STRUCTURE

Shuffling on this mortal coil

Roger H. Pain

It has been common knowledge for more than 20 years that some amino-acid residues have a greater tendency than others to take part in α -helix formation when incorporated into a polypeptide. But quantitative evidence is sparse and has been mainly based on the introduction of the residue under investigation as a 'guest' into a synthetic 'host'. In a new set of experiments, described on page 268 of this issue¹, Robert Baldwin and his colleagues have provided a natural context for the guest residue and so derive an order of helix-forming tendency more directly related to natural peptides in aqueous solution. As sometimes happens in well-designed experiments, the results are not entirely in keeping with expectation.

Regular secondary structure includes α -helices and is a feature of the folded three-dimensional conformation of most globular proteins. The length of the helices cannot be greater and is usually less than the diameter of the roughly spherical protein. One intriguing question is how an unstructured, randomly orientated polypeptide can fold into a reproducible conformation guided only by the information contained in the amino-acid sequence. Secondary structure elements are formed very early on in this folding process, followed more slowly by inter-residue packing and shuffling as the tertiary interactions build up^{2,3}. To understand the mechanism underlying this process, it is necessary first to predict which series of residues in a protein

sequence will form α -helices.

The frequency of any amino acid occurring in a helix has been analysed using a variety of known protein structures⁴. The value of this approach has been not so much in its moderate success at predicting the location of helices in new protein sequences, as in demonstrating that the tendency of a given residue to form part of an α -helix in a folded protein depends to a large extent on an intrinsic property of that amino acid. The fact that predictions are not always fulfilled suggests that tertiary interactions somehow moderate this predisposition. The statistical probability of helix formation derived from known sequences and structures is a complex of intrinsic tendencies and extrinsic interactions, and so cannot reliably predict the formation of helices in a run of amino acids at the stage when the protein is just beginning to fold, either *in vitro* or *in vivo*.

With the idea of keeping test residues in solution so as to measure their helicity or lack of it, an earlier approach was to incorporate them into a copolymer containing solubilizing residues such as hydroxypropyl-L-glutamine⁵. Results for these experiments led to the general view that small peptides such as poly-L-alanine could not form a stable helical structure in aqueous solution — a view that persisted until quite recently. It is now recognized that peptides can assume helical structure, although usually only at low temperatures.

With these concerns in mind, and against a background of meticulous work

1. Basho, M. *The Narrow Road to the Deep North and other Travel Sketches* (trans. Yuaso N.) (Penguin, London, 1965).
2. Smith, K.F. *et al. Phys. Lett.* **B234**, 191–196 (1990).
3. Smith, J. II Ph.D. thesis, Harvard Univ. (1951).
4. Altarev, I.S. *et al. JETP Lett.* **44**, 460 (1986).
5. Close, F. *Nature* **342**, 619 (1989).
6. Kobayashi, M. & Maskawa, T. *Progr. Theor. Phys.* **49**, 652 (1973).
7. Burkhardt, H. *et al. Phys. Lett.* **B200**, 169 (1988).
8. Patterson, J.R. *et al. Enrico Fermi Institute preprint EFI 90-95* (1990).
9. Flynn, J. & Randall, L. *Phys. Lett.* **B244**, 221 (1989).
10. Alitti, J. *et al. Phys. Lett.* **B235**, 363 (1990).
11. Abe, F. *et al. Phys. Rev. Lett.* **62**, 1825 (1989).
12. Ellis, J. & Fogli, G.L. *Phys. Lett.* **B232**, 139 (1989).
13. He, X.-G., McKellar, B.H.S. & Pakvasa, S. *Int. J. Mod. Phys.* **A4**, 5011 (1989).
14. Peccei, R. & Quinn, H.R. *Phys. Rev. Lett.* **38**, 1440 (1977).
15. Ellis, J. & Gaillard, M.K. *Nucl. Phys.* **150**, 141 (1979).
16. Gérard, J.M. *et al. Nucl. Phys.* **B253**, 93 (1985).
17. Weinberg, S. *Phys. Rev. Lett.* **63**, 2333 (1989).
18. Weinberg, S. *Phys. Rev. Lett.* **37**, 657 (1976).
19. Sakharov, A.D. *Pis'ma Zh. Eksp. Teor. Fiz.* **5**, 32 (1967).
20. Ellis, J. *et al. Nature* **293**, 41 (1981).
21. Kuzmin, V.A., Rubakov, V.A. & Shaposhnikov, M.E. *Phys. Lett.* **155B**, 36 (1985).

from Baldwin's group on the stabilization of the N-terminal helix of ribonuclease, Padmanabhan *et al.*¹ have constructed a series of poly-L-alanine-based peptides of the form CH₃CO-YKAAXAKA-AXAKAAXAK-NH₂. Features of these peptides are their blocked N and C termini, lysines (K) that confer solubility, and an N-terminal tyrosine (Y) that acts as a marker for concentration determination. They are similar in size to the helices in many folded proteins. Poly-L-alanine (A) peptides interspersed with lysine residues were already known to maintain a helical conformation in aqueous solution and the new peptide duly exhibited an average helix content of 78 per cent when the variable residue, X, was alanine. When X was leucine, the helix content was comparable, while peptides having X as isoleucine, phenylalanine or valine showed progressively reduced helicity. Even the peptide in which the two outer X residues were alanines, and the single X in the middle was valine, was appreciably less helical than when all three Xs were alanines, illustrating the destabilizing effect of valine.

As well as confirming that protein sequences of the appropriate composition can form stable helices at low temperatures (indeed, the folding of thermophilic proteins would pose an interesting extension of this work), there are two informative deviations from accepted wisdom. The first is that the helix-forming tendencies of different residues now extend over a wider range than was given by the

host-guest experiments due, at least in part, to the previously underestimated alanine contribution. Thus in the context of initiating protein folding, the early formation of helices is now expected to be much more sensitive to precise residue composition.

The second discrepancy is to be found in a comparison of the low helix-forming tendency of phenylalanine with the apparently strong helical preference evidenced by its situation in the middle of longer helices. The latter, if significant, must presumably reflect the participation of this residue in tertiary interactions, such as those stabilizing amphiphilic helices; this once more underlines the sophisticated differences in contributions from individual amino acids to protein stability, conformation and folding.

Five non-polar residues down, and 15 less-easily-treated residues to go — the 'simple' helix may yet have more surprises in store. □

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1. Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M. & Baldwin, R. L. *Nature* **344**, 268–270 (1990).
2. Udgaonkar, J. B. & Baldwin, R. L. *Nature* **335**, 694–699 (1988).
3. Roder, H., Elove, G. A. & Englander, S. W. *Nature* **335**, 700–704 (1988).
4. Schulz, G. E. & Schirmer, R. H. *Principles of Protein Structure* Ch. 6 (Springer-Verlag, New York, 1979).
5. Sueki, M. *et al. Macromolecules* **17**, 148–155 (1984).

METAZOAN PHYLOGENY

Reassessing relationships

Colin Patterson

STUDY of the higher level phylogeny of multicellular animals has been something of a backwater for decades, largely because all the morphological clues had been pushed beyond their limits, and mutually contradictory speculations led only to dead ends. But the field has recently become a centre of interest again, with a combination of molecular and palaeontological data leading to some radical new ideas. This excitement has been generated by the availability of a set of molecular sequences with an appropriate taxonomic spread. These are sequences of 18S (small subunit) ribosomal RNA (rRNA) from ten of the traditional metazoan phyla, and the first analysis¹ produced the surprising conclusion that metazoans are polyphyletic (Fig. 1a). That result was quickly criticized on grounds of inconsistency with 5S rRNA sequences² and morphological evidence^{3,4}, and the issue of metazoan polyphyly seems to be only an artefact of method^{5–7} (Fig. 1). But James Lake's new analysis of

the same 18S rRNA data⁸ has generated further surprising results (Fig. 2).

The major surprises in Fig. 2 are that of the two largest metazoan phyla, Arthropoda and Mollusca, the first comes out as clearly paraphyletic, and the second as possibly so. Paraphyly is a relative newcomer to the vocabulary of phylogenetics; not long ago a group had to be either monophyletic (good) or polyphyletic (bad). During the 1970s there was a controversy over whether arthropods are monophyletic or polyphyletic (see for example, refs 9,10). That controversy seemed settled in favour of monophyly, but no one had considered the possibility of arthropod paraphyly. A paraphyletic

group is a grade or ancestral taxon, like prokaryotes, invertebrates, fishes or apes. In terms of a tree, it contains some but not all of the descendants of a common ancestor, and in terms of characters it lumps together organisms lacking features of more derived descendants — membership is defined only by absence.

The interest of Lake's tree (Fig. 2) is not just in the novelty of the groupings it proposes, but in the method used to generate it. Three main methods have been used to get trees from sequence data: distance, parsimony and maximum likelihood. In 1987, Lake published¹¹ an ingenious method named 'evolutionary parsimony'. Unlike other methods, Lake's is immune to variations in evolutionary rate given one assumption: that in transversions from purine to pyrimidine either pyrimidine is as likely to be fixed as the other, and vice versa (if that assumption fails, the method weakens¹²). Unequal rates can generate artefactual trees through long (rapidly evolving) branches 'attracting' one another, and can lead to the paradox that the longer the sequences compared, the less likely it is that distances or parsimony methods will find the right tree¹³. Unequal rates are evident in the 18S rRNA data: over about 1,000 nucleotides, *Drosophila* has accumulated more than five times as many unique substitutions as has *Homo* or the earthworm, the millipede and sipunculid have about four times as many as that standard, whereas the polychaete worm and starfish show only about half as many⁵.

Lake's evolutionary parsimony has been applied to such vexed problems as the monophyly or paraphyly of Archaeobacteria^{14–16} and the relationship between humans and apes¹⁷. Although the generality of the method is controversial^{12,16}, its great advantage is that it permits statistical testing of the significance of the preferred tree; its great disadvantage is that it can be applied to four taxa only (Cavender¹⁸ has developed a modification applicable to

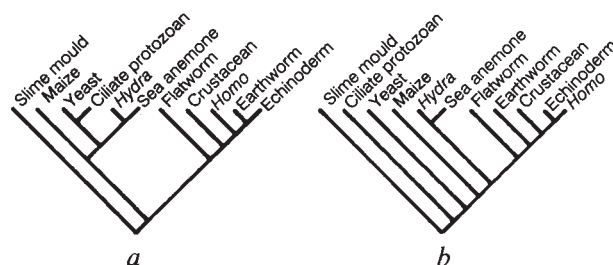


FIG. 1 a, Topology of 'polyphyletic Metazoa' tree generated by distance matrix analysis of 885 positions in 11 organisms selected from an alignment of 28 18S rRNA sequences¹. Metazoa are polyphyletic because the coelenterates are more closely related to a ciliate protozoan, a fungus (yeast) and a plant than to other metazoans. The tree is rooted on the slime mould *Dictyostelium*. b, Topology of shortest tree found by parsimony analysis⁹ of 544 variable positions in the same alignment as a. Metazoa are monophyletic, and the relationships shown match classical morphological ideas. As in a, the tree is rooted on the slime mould.

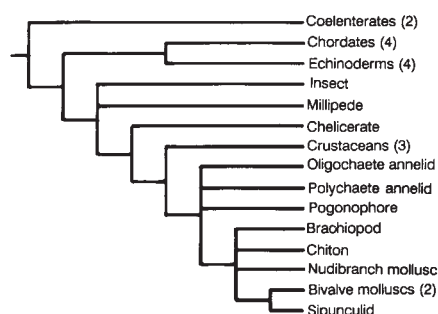


FIG. 2 Topology interpreted from two trees⁸ calculated by evolutionary parsimony from the same alignment of 18S rRNA as Fig. 1, with the addition of three crustacean sequences (a conchostracan, a notostracan and a pentastomid¹⁹). Numbers in brackets after group names indicate the number of species sampled. The tree is rooted on the coelenterates. In the published tree⁸ the three longest (most rapidly evolving) branches are *Drosophila*, the sipunculid *Golfingia* and the tunicate *Styela*, respectively with about 56, 55 and 37 transversions per 1,000 nucleotides. Echinoderms (a crinoid, an echinoid, a brittlestar and a starfish) did not emerge as monophyletic in the published tree.

more, but it has not yet been put to work). In many-taxon problems, Lake gets round this disadvantage by repeated sampling, calculating four-taxon trees, retaining all those with a central branch supported at the level of 5 per cent significance, and combining taxon pairs that are supported in every four-taxon tree containing them.

Lake's metazoan tree (Fig. 2) shows the Deuterostomia (Echinodermata + Chordata) as monophyletic, as does parsimony analysis of the same data^{5,6}. Because evolutionary parsimony deals only with unrooted trees (as do all methods of analysing sequence data, unless one roots on a particular taxon), Lake used coelenterates as the outgroup and looked for roots significant at the 5 per cent level. All five of those roots render arthropods paraphyletic, but one (Fig. 2) matches the traditional dichotomy between deuterostomes and protostomes. Within protostomes, this makes the two uniramiens (insects + myriapods) in this sample, individually or together, the sister-group of the remaining arthropods (Chelicerata, Crustacea) plus the annelids, pogonophore, brachiopod, molluscs and sipunculid. Further, the most probable position of the sipunculid is as the sister of bivalve molluscs, so rendering the Mollusca paraphyletic (molluscs did not emerge as monophyletic in any of the distance or parsimony trees¹⁶).

In the more distal part of the 18S rRNA tree, Abele and others¹⁹ have used Lake's method to confirm ($P < 0.04$) that the Pentastomida (tongue worms), parasites of tetrapod vertebrates usually given a phylum of their own, are modified crustaceans. Lake's tree might be read as indicating that in cladistic terms annelids, molluscs and the other minor phyla are

modified arthropods. Such an idea fits well enough with the evidence that molluscs are primitively metameric, but it does not fit traditional ideas on the development of the coelomic body cavity.

Annelid worms have a capacious coelom, as do vertebrates, but arthropods and molluscs have only rudimentary coelomic cavities, generally interpreted as reduced or vestigial. The major body cavity in arthropods is the haemocoel, a capacious blood circulatory system which in some arthropods and molluscs serves much the same function — a hydrostatic skeleton — as does the coelom in annelids. The most widely accepted schemes of coelomate history treat arthropods and molluscs as derived from annelid-like ancestors by reduction of the coelom of the circulatory system into a haemocoel^{5,20}. But Lake's tree implies that a haemocoel is primitive for protostomes (Fig. 2, insect and below) because haemocoelic animals branch off at each of the first three nodes.

In dealing with relationships as remote as those between arthropods and other phyla, we are certainly asking about events in the Precambrian, because arthropods, molluscs and brachiopods are all known in early Cambrian rocks. Interestingly, Valentine²⁰, in a recent analysis of late Precambrian metazoan trace and body fossils, concluded that the haemocoel preceded the coelom, that arthropods did not replace a capacious coelom but never had one, and that arthropods did not arise "from some plexus of annelid precursors, but that annelids arose from among a plexus of arthropod ancestors". Such ideas seem best tested by analysis of further sequences from molecules conserved since the Precambrian, and 28S (large subunit) rRNA is an obvious target. □

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- Field, K.G. *et al.* *Science* **239**, 748–753 (1988).
- Walker, W.F. *Science* **243**, 548–549 (1989).
- Bode, H.R. & Steele, R.E. *Science* **243**, 549 (1989).
- Ax, P. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 229–245 (Excerpta Medica, Amsterdam, 1989).
- Ghiselin, M.T. *Oxford Survs Evol. Biol.* **5**, 66–95 (1988).
- Patterson, C. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 471–488 (Excerpta Medica, Amsterdam, 1989).
- Lake, J.A. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 273–278 (Excerpta Medica, Amsterdam, 1989).
- Lake, J.A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 763–766 (1990).
- Manton, S.M. *The Arthropoda* (Clarendon, Oxford, 1977).
- Boudreaux, H.B. *Arthropod Phylogeny with Special Reference to Insects* (Wiley, New York, 1979).
- Lake, J.A. *Mol. Biol. Evol.* **4**, 167–191 (1987).
- Jin, L. & Nei, M. *Mol. Biol. Evol.* **7**, 82–102 (1990).
- Li, W.H., Wolfe, K.H., Soudis, J. & Sharp, P.M. *Cold Spring Harb. Symp. quant. Biol.* **52**, 847–856 (1987).
- Penny, D. *Nature* **331**, 111–112 (1988).
- Lake, J.A. *Nature* **343**, 418–419 (1990).
- Gouy, M. & Li, W.H. *Nature* **343**, 419 (1990).
- Felsenstein, J. *Nature* **335**, 188 (1988).
- Cavender, J.A. *Mol. Biol. Evol.* **6**, 301–316 (1989).
- Abele, L.G., Kim, W. & Felgenhauer, B.E. *Mol. Biol. Evol.* **6**, 685–691 (1989).
- Valentine, J.W. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2272–2275 (1989).

Catching the eye

DAEDALUS once invented a novel women's anti-wrinkle face cream, inspired by those spectacle lenses that darken in sunlight. Its fast-acting pigment lightened in the shadowed crevices of the wearer's face, but darkened on the smooth well-lit areas between them. This abolition of optical contrast made the wearer's wrinkles almost impossible to detect. Unfortunately, it also abolished perception of her facial style and expression. The new cosmetic turned the most vivacious woman into a sort of deadpan zombie. Bafflingly, it sold mainly to men — government spokesmen, psychiatrists, police interrogators, and others anxious to give nothing away.

So Daedalus is now turning the idea on its head. Some pigments, like thionine, amplify optical contrast: they are bleached by light, and darkened by shade. DREADCO's new 'Eyecatcher' cosmetics use this effect to enhance the optical contrast of a woman's face, giving her an appealing facial liveliness and dynamism. Contrast amplification is fast, but limited to the red region of the spectrum, so that blushing and other dynamics of complexion are emphasized, while the wrinkles are largely left alone. Daedalus is even formulating an Eyecatcher face cream for men, with a promise of added cragginess, stubblier stubble, and heightened moodiness of expression. And for actors, Eyecatcher grease-paint will convey the most subtle nuances of dramatic anguish to the cheapest and most distant seats.

Eyecatcher colours should also prove popular in clothing. Their exaggeration of contrast between highlight and shadow would subtly emphasize the contours and dynamics of the wearer's figure. This cunning anti-camouflage would call attention to the body far more beguilingly than conventional fluorescent colours. Joggers, exhibitionists, entertainers and their many imitators would welcome Eyecatcher clothing, and so would hikers or explorers setting out into dangerous wildernesses. Mothers, teachers and prison governors would also be keen to issue it to their more furtive and evasive charges.

In the form of paints and decorative material, Eyecatcher dyes would give a splash of added drama and impact to everything they touched. The depth of contrast that gives the sunlit tropics their sharp and almost passionate surreality, could be magically imported into a misty northern townscape or a quiet domestic interior. Eyecatcher varnish would bring out the surface detail of engineering components under inspection. And antique dealers would craftily apply it to their more battered offerings, to point up cracks, warping, grain, worm-holes and the other authenticating stigmata of great age.

David Jones

Avoidance of false positives

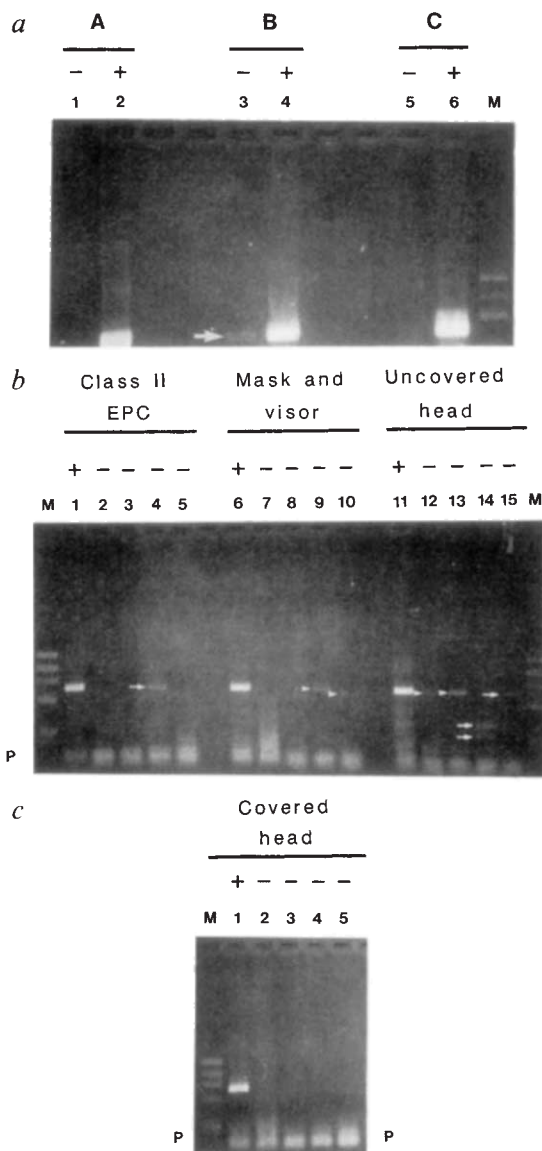
SIR—It is now widely accepted that great care must be taken to avoid false signals when amplifying nucleic acids by the polymerase chain reaction (PCR). One of the principal sources of contamination is the carry-over of material from one tube to another¹. But we have been able to trace a particularly alarming additional source of contamination to material, presumably dead epidermal cells, derived from the exposed facial skin surfaces of some of our PCR operators.

Over the past 18 months, we have developed and now routinely apply a diagnostic assay for proviral simian immunodeficiency virus (SIV) in experimentally infected macaque monkeys². We initially found false positives arising in co-processed negative controls, or from control animals, but were able to eliminate such occurrences by strict adherence to the principal recommendations of Kwok and Higuchi¹.

A few months ago, in spite of our meticulous precautions, operator B began to experience repeated problems with false positives. Panel *a* shows the results obtained when three separate individuals set up identical PCR assays in the order A, then B, then C. All used the same reagents and equipment. Operator B obtained a false positive result (white arrow, track 3) yet operator C, who used all the same reagents immediately after they had been handled by operator B, did not (track 5). The negative controls (panel *a*, tracks 1, 3, and 5) contain all the components of the PCR assay except for the added template and are sealed before removal from the "clean" laboratory for amplification elsewhere. We have not been able to attribute these results to any obvious differences in technique or any other factor peculiar to operator B (including HIV or SIV antibody status).

Three weeks later, after operator A had replaced every reagent used for the PCR in our "clean" laboratory, operator C also began to obtain false positives. It seemed increasingly likely that the problem was personally attributable to operators B and C, since operator A continued, and continues, not to experience such problems.

Accordingly, operator C then conducted a series of experiments based on this assumption, the results of which are shown in *b* of the figure. PCR assays were



All panels show the products of a standardized SIV *gag* gene PCR (see Kitchin *et al.*, 1990) after agarose gel electrophoresis. The SIV specific product size is 760bp (tracks labelled +). This product hybridizes to a specific, internal, radiolabelled oligonucleotide and has been fully sequenced to confirm its identity. Tracks labelled (–) are assay-mix only, negative controls, that do not contain experimentally added exogenous template. P marks the position of the primers or very short non-specific PCR products. M is ϕ X174 DNA digested with HaeIII. White arrows mark the false positive PCR products.

set up in groups of five (1 positive and 4 negatives) under conditions designed to offer different degrees of protection for the work from the normally exposed facial skin surfaces of the operator. In all the experiments, the operator wore a clean laboratory coat and gloves which sealed its cuffs. When the assays were set up in a

class II Exhaust Protected Cabinet, 1 out of 4 negative controls was positive (white arrow tracks 2–5), but when the assays were set up on the open bench with the operator wearing a disposable particle mask (3M Ltd, UK) as face protection and a clear plastic visor covering the rest of the face, but no face cover, 2 out of 4 false positives were observed (white arrows tracks 7–10). One of these products (track 10) was too small to be a specific PCR product derived from SIV. Confirmation that the source of the contamination was the exposed head and face of operator C was provided by the results shown in tracks 12–15; the open tubes were deliberately contaminated by shaking operator C's exposed head and hair over them; all 4 negative controls were positive (arrows, lanes 12–15).

These results show that some people can pick up DNA contamination on their skin surface which can then seriously compromise a PCR assay unless there are sufficient and appropriate negative controls. We believe that operators B and C probably acquired this contamination by simply entering the post-PCR, template preparation, or the cloning laboratories. The problem of contamination became noticeable only after we had considerably increased the sensitivity of our PCR assay. The present assay is capable of detecting about 10 copies of the SIV *gag* gene in the presence of DNA from 50,000 uninfected macaque lymphocytes.

It is relatively easy to control this problem by wearing appropriate protective clothing that prevents, or significantly reduces, the shedding of material from the head and face. The PCR assays shown in *c* of the figure, were set up by operator C on the open bench. Operator C's hair was covered by a disposable mob cap (Pal Wear Ltd, UK), and the face by a disposable mask (as above) as well as a pair of safety goggles. All the negative controls were negative (tracks 2–5).

Operators B and C have now routinely processed samples at all stages of the PCR, without experiencing further false positives. These protective items are cheap, easily obtainable, disposable (except the goggles) and reasonably comfortable to wear. For institutions that may not have the luxury of 3 or 4 separate laboratories and a separate technician for each stage of the PCR, these precautions will considerably reduce contamination at all stages.

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1. Kwok, S. & Higuchi, R. *Nature* **339**, 237–238 (1989).
2. Kitchin, P.A. *et al.* in *Animal Models in AIDS*, (Elsevier/North-Holland Biomedical, 1990).

Scaling of sexual activity

SIR—Anderson and May¹ observe a striking approximate relationship of the form $\sigma^2 = a\mu^b$ between the variance (σ^2) and the mean (μ) of the number of sexual partners taken per unit of time, in surveys of many different types and in a variety of populations and locations, but so far, there has been no explanation.

We use a simple probabilistic model of a closed homosexual population to show that such scaling laws may arise naturally as a result of pair formation and dissolution. Our analysis suggests that a scaling exponent close to four may be the norm at very low levels of activity (which means infrequent pair formation and dissolution). Stochastic simulations provide some justification for the view that the observed exponent ($b=3.231$) is the result of increased variability at slightly higher mean levels.

We assign to the i th individual unique values s_i , and f_i , the probabilities per unit time of that individual initiating dissolution of a pair (if paired), and of seeking a new partner (if single). The $\{s_i\}$ and $\{f_i\}$ are drawn from some given distribution on the interval (0,1). At each time step, the sequence of events is: (1) separation of (some) pairs, the individuals returning to the singles pool; (2) each single individual "decides" whether or not to look for a new partner; (3) individuals who are looking are paired off at random (in our simple model). No changes occur until the start of the next time interval.

The asymptotic rate of acquisition of new partners in the model is $r(F,S) = (1/F + 1/S - 1)^{-1}$, where F and S are the asymptotic averages of pair formation and separation rates in the populations of singles and pairs respectively. Their exact form will depend on model assumptions and need not equal the equivalent statistics of $\{s_i\}$ and $\{f_i\}$ for the population as a whole.

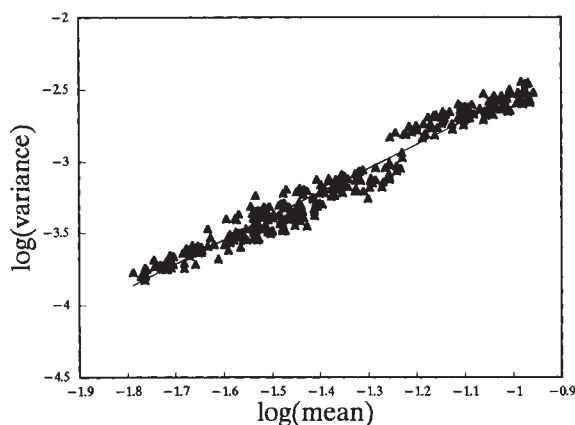
Expanding $r(F,S)$ around the value $\bar{r} = r(\mu_F, \mu_S)$, where μ_F and μ_S are the expected values of F and S respectively (see ref. 2), we obtain approximate expressions for the mean and variance of the rate of new partner acquisition as

$$\begin{aligned}\mu_r &= \bar{r}(\alpha\bar{r}^2 - \beta\bar{r} + 1), \\ \sigma_r^2 &= \alpha\bar{r}^4,\end{aligned}$$

where $\alpha = \sigma_F^2/\mu_F^4 + \sigma_S^2/\mu_S^4$ and $\beta = \sigma_F^2/\mu_F^3 + \sigma_S^2/\mu_S^3$, with σ_F^2 and σ_S^2 the variances of F and S respectively. Covariance terms are

dropped because, in our model, there is no correlation between the $\{s_i\}$ and the $\{f_i\}$. The equations indicate a fourth order scaling of variance with mean as $\bar{r}$ tends to zero, for small enough α and β . A scaling 'law' such as Anderson and May report can arise where the values of F and S observed in the various samples are not large and the variances associated with them are moderate.

To test the validity of this approximation, we carried out a stochastic simulation of our model with various population



Scaling law by simulation where log variance and log mean of number of partners acquired per unit time are approximately related by $y = -0.869 + 1.675x$; $r^2 = 0.9624$, $n = 299$.

sizes and distributions from which the $\{s_i\}$ and $\{f_i\}$ were randomly drawn. The figure illustrates how a scaling law can arise. In this case, the $\{s_i\}$ and $\{f_i\}$ were drawn from identical Beta distributions, with mean values 0.035 to 0.167 and variance 0.0011 to 0.0107. The resulting scaling relationship between mean and variance of numbers of partners per unit time (fitted line in figure) is of the form $\sigma_r^2 = a\mu_r^b$ with $a = 0.41$, and $b = 1.67$. It is possible to find many scaling patterns with different exponents. We speculate that the exact value noted by Anderson and May reflects a restrictive set of population parameter values. We are looking at other mixing and pair formation/dissolution models in order to determine whether or not "realistic" mixing structures give rise to scaling patterns similar to that reported.

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1. Anderson, R.M. & May, R.M. *Nature* **333**, 514–519 (1989).
2. Mood, A.M., Graybill, F.A. & Boes, D.C. *Introduction to the Theory of Statistics*, 3rd Edn (McGraw-Hill, New York, 1974).

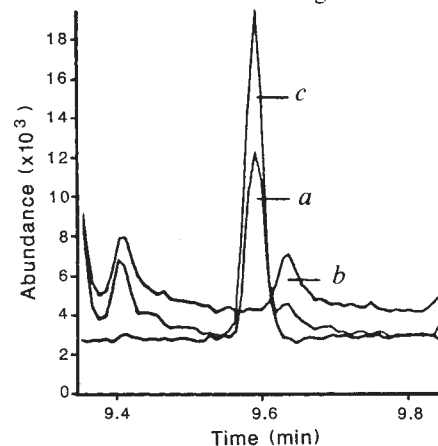
Irradiation detection

SIR—Methods of distinguishing between irradiated and unirradiated foodstuffs would be useful to enforcement authorities and would increase the confidence of consumers in food irradiation.

Radiation-induced free radicals in bone can be detected by electron spin resonance spectroscopy¹ and irradiated herbs and spices identified by thermoluminescence², but many irradiated foods are not amenable to these methods. Thus there is a need to extend the range of methods available. Irradiation of simple triglycerides at high dose (60 kGy) yields cyclic compounds known³ to be substituted cyclobutanones with as many carbon atoms as the parent fatty acid and with the alkyl group at ring-position 2. Our interest is to use these compounds as indicators of irradiation in complex foods at doses well below the recommended maximum of 10 kGy.

It was decided to concentrate on one compound, 2-dodecyl cyclobutanone, formed from palmitic acid. An authentic sample was synthesized and characterized using NMR, infrared spectroscopy and mass spectrometry. We have worked with irradiated minced chicken, in which about 25 per cent of total carcass fatty acids are in the form of palmitic acid.

A peak has been consistently identified from irradiated chicken meat using GC-MS with selected monitoring of ions 98



Selected ion monitoring of the sum of ions 98 and 112 from: a, 2-dodecyl cyclobutanone standard; b, an extract of a 10-g sample of non-irradiated; and c, irradiated (5 kGy) minced chicken meat. The 2-dodecyl cyclobutanone was extracted using diethyl ether and the extract evaporated to dryness before dissolution in acetonitrile saturated with hexane. Calcium stearate was added and the mixture shaken. The hexane layer was removed, taken to dryness under nitrogen and resuspended in 1 ml hexane containing internal standard. The sample was analysed using a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 mass selective detector. An Ultra 1 column was used in the GC.

and 112. The former constitutes the most abundant peak in the mass spectrum of the authentic standard. The ion ratio and retention time are identical to those of the

authentic butanone. Unirradiated samples show no detectable peak at this retention time under our analytical conditions. The peak is reproducible, and can be detected 20 days after irradiation.

We have established that the concentration of 2-dodecyl cyclobutanone produced by 5 kGy irradiation of minced chicken meat is approximately $0.2 \mu\text{g g}^{-1}$ fresh weight, five times our estimated limit of detection.

Our technique shows that 2-dodecyl cyclobutanone is a potential post-irradiation marker for minced chicken meat and possibly for other products. Further work is needed to tell whether the technique can be used to estimate irradiation doses and whether the marker survives various processing and storage conditions.

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1. Stevenson, M.H. & Gray, R. J. *Sci. Food Agric.* **48** 269–274 (1989).

2. Sanderson, D.C.W., Slater, C. & Cairns, K.J. *Nature* **340**, 23–24 (1989).

3. Letellier, M.H. & Nawar, W.W. *Lipids* **7**, 75–76 (1972).

Filters for cell culture

SIR—Knight (*Nature* **343**, 218; 1990) recently highlighted adverse effects of disposable filters on cell culture. We are encouraged to see that the performance of filters has been questioned. Investigators often do not realize that the use of such devices can lead to the addition of unknown variables to their procedures. However, this does not necessarily apply to all filters.

The wetting of polymeric filtration membranes with polyglycol ethers or alternatives is generally necessary for filter preservation or their use with aqueous solution. These wetting chemicals are commonly leached from such membranes when incorporated in disposable syringe filters and other filtration devices. Leached chemicals may well interfere in many applications, especially cell culture.

On the contrary, inorganic membranes do not require the addition of such chemicals during their manufacture. We are concerned that the title of the correspondence may lead some scientists to believe that all membrane types leach such wetting agents and additives, which is not the case.

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Tropical leaf-litter nutrients

SIR—Singh *et al.*¹ suggest that microbial biomass is the main source of plant nutrients in dry tropical forest and savanna, but sources other than microbial biomass, and especially litter, could be of considerable importance in these ecosystems.

The annual rates of litter fall range from 5 to 10 tons per hectare in tropical forests. Unlike timber and roots, litter is frequently high in nitrogen. There is appreciable net mineralization as a result of its decomposition. Studies in Zaire and Guatemala have shown that about 80 per cent of potassium, 58 per cent of phosphorus and 38 per cent of nitrogen in the litter are mineralized within 4 weeks², thereby releasing 30 to 75 kg nitrogen, 1 to 4 kg phosphorus, and 5 to 50 kg potassium per hectare.

Litter accumulates largely during winter and summer, and the nutrient release of the accumulated biomass peaks at the onset of monsoon, when the rate of mineralization is high. Regardless of the source, mineralization of organic nitrogen increases sharply when dry soils are moistened^{3,4}, so the high release of nitrogen at the onset of monsoon need not be attributed exclusively to microbial biomass.

My opinion is further strengthened by recent observations⁵ that, in the oak savanna of the foothills of the Sierra Nevada in a mediterranean climate, nitrogen in litter decreased fastest during late autumn and early spring, coinciding with the peak periods of plant growth. Through the early spring (February to April), nitrogen in litter decreased by about 47 per cent, compared with a decrease of roughly 7 per cent of nitrogen in microbial biomass, signifying that litter may make a larger contribution than microbial biomass to plant nutrition in the three ecosystems studied by Singh *et al.*

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1. Singh, J.S., Raghubanshi, A.S., Singh, R.S. & Srivastava, S.C. *Nature* **338**, 499–500 (1989).

2. Sanchez, P.A. *Properties and Management of Soils in the Tropics* (Wiley, New York, 1976).

3. Kundu, D.K., thesis, (Indian Agr. Res. Institute, New Delhi, 1987).

4. Tusneem, M.E. & Patrick, W.H., Jr. *Bull.* **657**, (Agr. Exp. Stn., Louisiana State Univ., 1971).

5. Jackson, L.E., Strauss, R.B., Firestone, M.K. & Bartolome, J.W. *Plant and Soil* **110**, 9–17 (1988).

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

The name game

SIR—Bremer *et al.*¹ enjoy biologists to welcome frequent changes of nomenclature because they reflect constant improvements in knowledge of ancestral relationships. We explicitly reject the view that taxonomists have a responsibility to supply the stable nomenclature that most biologists need, and go on to suggest that synonyms should be accessible as an international database.

Biologists find it difficult enough to keep up to date with publications that are strictly relevant to their own research and do not welcome the additional burden of having to make constant cross references to the ever-increasing lists of synonyms. Few non-taxonomists see why research on ancestral relationships cannot proceed without accentuating the nomenclatural confusion.

The eminent systematist, Ernst Mayr, has assured us² that "... there is a large minority (perhaps actually a majority) of taxonomists who are as anxious to retain the stability of names as the average biologist. These ... taxonomists fully realize that names are the keys to a vast information storage and retrieval system and that every change of name corresponds to a change of a key, resulting in confusion and loss of information."

Bremer¹ suggests that Mayr has been over-optimistic; sadly, this is certainly true of those taxonomists who work with yeasts, as can be seen from the following examples from recent work of some of the most distinguished researchers in this field. (1) In 1984, Kurtzman published descriptions of 30 species of *Hansenula*³ and yet, the same year, he abolished almost the whole genus⁴. (2) In a series of papers published in 1987, Nakase and his colleagues described^{5–7} four new species of *Sporobolomyces* which, only the following year, he moved to the genus *Bensingtonia*⁸. (3) However, van de Walt has outdone even these authors, describing the new species *Sporobolomyces phylladus*⁹ after having previously changed its name to *Bensingtonia phylladus*¹⁰.

1. Bremer, K., Bremer, B., Karis, P.O. & Källersjö, M. *Nature* **343**, 202 (1990).

2. Mayr, E. *Nature* **339**, 654 (1989).

3. Kurtzman, C.P. in *The Yeasts: a Taxonomic Study* (ed. Kreger-van Rij, N.J.W.) 165–213 (Elsevier, Amsterdam, 1984).

4. Kurtzman, C.P. *Antonie van Leeuwenhoek* **50**, 209–217 (1984).

5. Nakase, T. & Suzuki, M. *Trans. Mycol. Soc. Japan* **28**, 1–8 (1987).

6. Nakase, T. & Suzuki, M. *J. gen. appl. Microbiol.* **33**, 177–196 (1987).

7. Nakase, T., Suzuki, M. & Itoh, M. *J. gen. appl. Microbiol.* **33**, 445–454 (1987).

8. Nakase, T. & Boekhout, T. *J. gen. appl. Microbiol.* **34**, 433–437 (1988).

9. van der Walt, J.P. *et al.* *Antonie van Leeuwenhoek* **55**, 189–195 (1989).

10. Yamada, Y., Nakagawa, Y. & van der Walt, J.P. *Agric. biol. Chem.* **52**, 3203 (1988).

11. Dixon, M. & Webb, E.C. *Enzymes* 2nd Edn (Longman, London, 1964).

One of two procedures might make things easier. First, each species could be given both a very constant official recommended trivial name as well as taxonomically respectable, and hence fickle, systematic name. Such a formal dual system of nomenclature has been used for enzymes¹¹. Alternatively, acceptance of new names could await official approval of an international commission.

The need for a stable nomenclature is so great that its dismissal by many taxonomists often leads to scorn and derision by experimentalists and other biologists. The remedy for this sad state of affairs is in the hands by the taxonomists themselves.

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Direct repeats in HSF binding sites

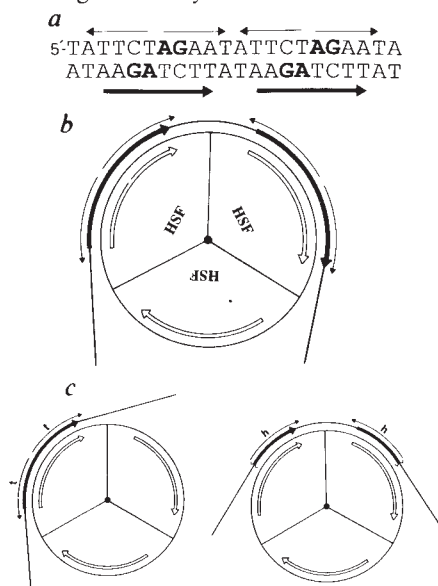
SIR—Two recent articles show that heat-shock factor (HSF) is a trimer both in solution and when bound to DNA^{1,2}, which strongly suggests that the monomers form a cyclic arrangement (with symmetry group C_3), and in particular that the DNA binding site on each monomer is related to its neighbour by a rotation of 120°. In connection with certain prokaryotic transcriptional activators, it has been noted³ that such an arrangement is more easily compatible with a DNA binding site consisting of directly repeated nucleotide sequences than of a palindromic sequence.

Although HSF binding sites have been described⁴ as the palindromic sequence —CT-GAA-TTC-AG-, further investigation has shown that the situation is more complicated, and that -GAA- boxes (thin arrows in *a* of figure) in direct and inverse orientation play an essential role in binding^{2,5}. From inspection, it is easy to see that the binding site can also be regarded as consisting of two directly repeated sequences (bold arrows) centred on the four nucleotides that directly contact the DNA². The distance between the centres of the repeats is 10 base pairs (one helix turn), so that the motifs can be accommodated on the same face of a DNA helix.

The quaternary structure of the oligomer and this alternative view of the structure of the binding site naturally suggest the model shown in *b* of the figure, which can account for several surprising observations. Thus *c* of the figure is one plausible explanation of the reasons why HSF interacts equally well with the so-called tail-to-tail motifs and head-to-head motifs². Moreover, the decreased mobility in polyacrylamide gels of the complex formed between HSF and increasing numbers of -GAA- boxes may be at least partly accounted for by a progressive

increase of the bending of the DNA fragment as it is wrapped around the protein rather than by an increase of the numbers of HSF trimers bound to the DNA. (Bending induced by HSF has already been observed⁶.)

If this model is correct, there may be a striking similarity between the recent



a, Nucleotide sequence consisting of an array of two 5bp inverted units (thin arrows) as used in *in vitro* studies² and the alternative DNA binding site (bold arrow) for a HSF monomer now suggested. The nucleotides shown in bold type are those probably in contact with the protein². *b*, Interaction between an HSF trimer and the nucleotide sequence of the figure. The open thick arrow represents the monomer binding site. (Note that HSF trimers may form multimers under some conditions¹.) *c*, Suggested interaction of HSF with inverted 5bp units in tail-to-tail (t-t) and head-to-head (h-h) configurations, as described in ref. 2.

histories of the prokaryotic transcriptional activator AraC and HSF. In both cases, the DNA binding site was at first described as a degenerate palindromic sequence on the basis of nucleotides sequence and footprinting^{7,8}. But the AraC binding site was subsequently shown to be made up of directly repeated sequences which are also perfectly compatible with the footprinting data^{9,10}. I propose that there will be a similar outcome for HSP.

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1. Sorger, P.K. & Nelson, H.C.M. *Cell* **59**, 807–813 (1989).
2. Persic, O., Xiao, H. & Lis, J.T. *Cell* **59**, 797–806 (1989).
3. Raibaud, O. *Mol. Microbiol.* **3**, 455–458 (1989).
4. Pelham, H.R.B. *Cell* **30**, 517–528 (1982).
5. Amin, J., Ananthan, J. & Voeltz, R. *Mol. Cell Biol.* **8**, 3761–3769 (1988).
6. Shuey, D.J. & Parker, C.S. *Nature* **323**, 459–461 (1986).
7. Shuey, D.J. & Parker, C.S. *J. biol. Chem.* **261**, 7934–7940 (1986).
8. Hendrickson, W. & Schlieff, R. *Proc. natl. Acad. Sci. U.S.A.* **82**, 3129–3133 (1985).
9. Brunelle, A. & Schlieff, R. *J. mol. Biol.* **209**, 607–622 (1989).
10. Lee, N., Francklyn, C. & Hamilton, E.P. *Proc. natl. Acad. Sci. U.S.A.* **84**, 8814–8818 (1987).

A more balanced view

SIR—Salter is undoubtedly on the right track when he suggests in his News and Views article (*Nature* **343**, 509; 1990) that the recent anomalous gyroscope results of Hayasaka and Takeuchi (*Phys. Rev. Lett.* **63**, 2701; 1989) are due to vibration. The weighing of non-steady loads by means of simple mechanical or electronic balances must be one of the most fundamental experimental errors.

Unfortunately, Salter's explanation for the strong dependence of the vibration (and thus anomaly) upon spin direction is less convincing. There is a simple classical explanation for this aspect; one which would have been all too familiar to Lord Kelvin's students. Kelvin was wont to give a student a horizontally spinning gyroscope, held in a tray-like frame, and ask the victim to imagine that he was serving imaginary guests in one direction, there was no problem. If he turned back the other way, the 'tray' would convulsively try to invert itself and sometimes injure the holder.

The reason is not hard to find. The stable angle for the swung gyroscope is proportional to a term of the form, $A/(B+C)$, for one spin direction and to a term of the form $A/(B-C)$, for the other spin direction. If B and C are similar in magnitude, the difference in the terms can be significant. The relevance of this is that, in the Japanese experiment, the Earth's surface becomes the tray and the Earth's rotation is equivalent to the student's swinging motion. I surmise that, when the gyroscope is spinning in one direction, it is fairly stable, the spin axis remains vertical, there is no precession or nutation (it is 'sleeping'), and hence no vibration. When it is spinning in the other direction, the interaction with the Earth's spin causes a deviation from the vertical (like a budgerigar swinging its cage), appreciable precession and nutation, and thus enough vibration to cause the apparent anomaly.

The gyroscope/Earth-spin interaction seems to have been first studied by Sire (*Arch. des Sci. Phys. Nat. Genève* **I**, 1858), and Gilbert's barogyroscope (which had essentially the same form as the Japanese apparatus, apart from the balance) was designed to demonstrate the Earth's rotation. On a more mundane level, north-seeking gyroscopic compasses used to have mechanisms which corrected for errors that depended upon the latitude position and the ship's speed. Perhaps the absence of any anomaly in later experiments was due to the use of apparatus for which $B \gg C$.

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Gorillas in the midst

Jonathan Marks

Primate Origins and Evolution: A Phylogenetic Reconstruction. By R. D. Martin. Chapman and Hall: 1990. Pp.804. £89, \$125.

BIOLOGICAL anthropology is a field distinctive for its curious attitude towards dilettantism. In most scientific disciplines, dilettantism is flatly abjured, yet in biological anthropology the proffered opinions of ornithologists or fruitfly geneticists (among whom, let me say, I number some of my best friends) are sometimes taken as authoritative. There are even university textbooks written solely by journalists. As far as I know, this is not the case in organic chemistry or endocrinology.

Biological anthropology is of course an immensely broad field, in which researchers with such diverse specializations as ethology, anatomy and genetics are united by virtue of their subject matter, the primates — and who therefore find it difficult to evaluate and appreciate each other's work. Perhaps that is why they are generally less reluctant to embrace the opinions of variably informed outsiders. Further, for the past 25 years, no synthetic work on the subject has been available; generating such a volume would be an immense undertaking, and few have the requisite magisterial scope of knowledge.

R. D. Martin of the University of Zurich, a distinguished student of the primates, has attempted to bridge these intellectual islands. The result is emphatically not the work of a dilettante. Martin's book summarizes modern views of evolutionary relationships among primates in remarkably clear language. *Primate Origins and Evolution* is a comprehensive, unified work on the biological context of our species, in the tradition of Wilfrid E. Le Gros Clark's *The Antecedents of Man* (1959) and John Buettner-Janusch's *Origins of Man* (1966). The book is in my opinion destined to take a place among the chief scholarly works of this generation.

Martin's book has only one modern rival, John Fleagle's excellent *Primate Adaptation and Evolution* (Academic, 1988). But the two are rather more complementary than antagonistic: Fleagle's

is more of a text, Martin's more of a reference; Fleagle's is organized taxonomically, Martin's by biological system; the evolution of *Homo sapiens* is the climax of Fleagle's book, but is only a minor diversion in Martin's text.

IMAGE
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Red Pata monkeys, doing what comes naturally.

Martin begins with the principles of phylogenetic reconstruction, then applies them to the teeth, guts, head, gonads, limbs and proteins of various primates. The key phylogenetic dichotomies he addresses are between primates and tree shrews, archaic and modern primates, prosimians and anthropoids, lemurs and lorises and, to a lesser extent, humans and apes. But Martin is not content merely to summarize data — he analyses and criticizes as well, unafraid of taking a stand and wagging a finger.

Martin's approach to systematics, for example, is cladistic in analytical method, but eclectic in classification. Thus he allies tarsiers with monkeys, apes and humans phylogenetically, but prefers to classify them with the lemurs and lorises; similarly, he allies gorillas and chimpanzees

with humans phylogenetically, but classifies them with orang-utans. His lucid justifications for these conclusions should be mandatory reading for students.

Some of Martin's stands are contentious, yet the bases for his judgements are given clearly, implicitly daring the reader to disagree. There is an underlying polemic to Martin's work — as there is to all significant science — here especially concerning the place of tree shrews in relation to primates. For half a century, following Le Gros Clark, primate biologists accepted tree shrews either as among the primates, or at least as their sister taxon. Martin reviews the evidence for this judgement and rejects it — but there certainly is an awful lot of homoplasy involved in that decision.

Planet Earth Pictures

Although he clearly describes the principles of phylogenetic reconstructions and modern evolutionary theory, Martin is occasionally inconsistent in appreciating their implications. He is sceptical, for example, of the traditional association of "archaic" Palaeocene Plesiadapiformes with Euprimates (see F. Szalay, A. Rosenberger and M. Dagosto, *Yb. phys. Anth.* 30, 75; 1987), as the former have few or none of the skeletal characteristics that we have come to expect of primates. Yet they share similarities of the molar teeth with early Euprimates, especially the Eocene *Pelycodus*. A simple solution, rejected by Martin, would be that molar teeth were among the first of the primate suite of features to develop; that they were present in archaic primates

before the characteristic specializations of the skull and extremities emerged; that a radiation in the late Palaeocene involved the rapid emergence of these features in Euprimates from Plesiadapiformes or something like them; and that the molar teeth were coincidentally retained by the specific lineage of which *Pelycodus* was a part.

Martin's interpretation, rather, takes the Plesiadapiformes and the Euprimates independently back to a data-free mid-Cretaceous limbo. This hypothesis may of course be correct; but it is necessary only if one rejects the possibility of dental retention in the *Pelycodus* lineage, and as well the possibility that the main characteristics (and presumed adaptations) of the Euprimate radiation could have evolved very rapidly. I tend to think they

could have, following the majority, and not having an insider's knowledge of the palaeontology, in particular of the basicranium, which Martin dismisses. Whether these characteristics actually did develop quickly is another matter, but it certainly took a very few million years for hominids to radiate from an ape stock — why should it have been much longer for Euprimates to emerge from archaic primates?

Martin's chapter and a half on primate molecular evidence provides a competent and critical review, yet most of the issues he raises are moot, as he focuses almost exclusively on protein data. Martin's broad criticisms of many assumptions of molecular evolutionary analyses seem to be sound, and the section stands as a thoughtful summary of the state of molecular systematics before nucleotide sequencing became common. Martin puts somewhat more faith than me in the idiosyncratic analysis of chromosomal R-bands, and in a DNA hybridization analysis of lemurs published in 1980,

which drew conclusions from small differences in the extent of DNA renaturation, rather than from differences in the thermal stability of the DNA hybrids.

The book is illustrated skillfully by the line drawings of Anne-Elise Martin. My only criticism of the general scope of the work, and it is a minor one, is that only in the first chapter are we shown primates as whole entities. Throughout the rest of the book they are presented in bits and pieces — skulls, teeth, chromosomes — which tends to give most of the volume a cold and analytical feeling. What is largely lacking is a presentation of primates *alive*: eating, jumping, metabolizing, reproducing, so brilliantly captured in the illustrations by Nash and others in Fleagle's book. But for this minor criticism, Martin's book remains an extraordinary accomplishment, a monumental synthesis which I anticipate will be a standard work for years to come. □

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The future perfect

Brian Bertram

Conservation for the Twenty-First Century.

Edited by David Western and Mary Pearl. Oxford University Press: 1989. Pp.365. £30, \$36.95.

"CONSERVATION 2100" was a symposium held in October 1986, convened by that admirably wide-ranging organization, the New York Zoological Society. The 32 authors of the papers in this resulting volume have in common considerable expertise in fields relevant to the conservation of wildlife. They were asked to take a long view: they have done so, and collectively a very broad one as well. A well-edited collection of thoughtful, quotable papers is the result, which makes a valuable contribution to the debate on how to tackle the problems of conservation.

Predictions are always difficult — especially about the future. One relatively optimistic example here is that by the year 2100 the world's human population will have levelled off to between 8 and 13 thousand million people, with growth rates being very uneven in different parts of the world. There are also some appalling projections, such as a Mexico City of 30 million inhabitants. The assumption is that population control will be correlated with a rise in living standards, and the point is forcefully made that "the problem of excessive population growth will not be solved in the uterus but in the human

mind". It is considered that "producing enough food will be a trivial problem late next century when population levels off". This will be possible mainly by increasing agricultural production by intensification rather than by expansion of agricultural land — indeed it is postulated that much land at present cultivated may become available for wildlife. It is envisaged that nature will "emerge from an environmental bottleneck late in the twenty-first century".

This apparently orderly situation does of course depend on relative stability in most of the world, an absence of large-scale catastrophes, major wars or wide-scale civil unrest. These may not be wholly realistic assumptions, but it is scarcely possible to incorporate the alternatives into sensible planning.

In this human-pressurized world of the future, the growing field of conservation biology will become increasingly important. The rate of extinction of species is accelerating, caused by the 'evil quartet' of overkill, habitat destruction, introduced species and chains of extinction. It is estimated that by the turn of the present century, human activities could have wiped out 15–25 per cent of all species. We need to know much more about the evolutionary potential of organisms — how species and genes adapt and evolve in changing circumstances. Natural communities and ecosystems are highly complex, and the loss of some species may have dramatic effects on the ecological balance within an ecosystem.

Management of nature will become increasingly necessary, indeed inevitable. Strong cases are made that it should be

carried out not only inside the limited protected areas which will persist, or outside such areas in some form of relationship with human activities, but also *ex situ* in zoos, botanical gardens, artificial reserves and refrigerators. The agenda for the future calls for great improvements in all these areas — for an expansion of protected land, for corrective management, for integrated land use that incorporates both human and wildlife interests, and for more and better *ex situ* methods.

But "the extinction crisis has reached the point where it is no longer possible for thoughtful people to consider the situation manageable by professional wildlife managers and park professionals alone. In order to conserve significant diversity in the earth's biological resources beyond the year 2100, action is needed by society as a whole". It must be recognized that without some action our downhill slide will continue, resulting not just in the disappearance of some 'nice' animals, but in the weakening of the whole ecological basis of our existence.

The conservation agenda calls for a broadened mandate: "conservation is seen now as just one more in a varied group of special-interest lobbies. The task before conservationists is a transformation from that status to one of a social force capable of bridging and uniting diverse interests by identifying joint solutions to common problems". Conservation is not seen as opposed to development; rather, both are essential, because "the one 'pollutant' that threatens above all others to destroy our environment . . . is the permanent, pervasive condition of poverty". Western and his colleagues believe that "one immediate task is to address international economic imbalances", and that conservation has to be linked with improvements in human welfare.

If these selected points sound naive to some, the book as a whole most emphatically does not. It is written by realists who know what they are talking about. Since the 1986 symposium, there have been dramatic changes in attitudes throughout the world, both an overturning of apparently accepted values in eastern Europe and a far wider appreciation of the realities of impending climatic change. Attitudes and aspirations can change fast. We have to learn how to limit individual hopes, and how a more affluent population in a free society can be educated to refrain from making excessive use of the world's resources. And we have to hope that it is not naive to be prepared to work towards such changes in thought in the century ahead. □

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Thought for food

Johannes Friedrich Diehl

Biology of Food Irradiation. By David R. Murray. *Research Studies Press, 24 Belvedere Road, Taunton, Somerset TA1 1HD, UK: 1990. Pp.255. £25.*

The general public's knowledge about food irradiation is minimal, and a good review intelligible to "anyone who is concerned about the quality of the food they eat" would be most welcome. The text on the back cover of the *Biology of Food Irradiation* promises this, adding that "the effects of food irradiation are disastrous". This foregone conclusion dominates the book from the beginning to the end. In his eagerness to unmask food irradiation as an irresponsible plot against the health of unwary consumers, the author has thrown all objectivity overboard.

Murray starts, appropriately enough, with a definition of radiation dose and dose units, but then embarks on a rambling account of Gregor Mendel and his breeding experiments with differently coloured flowers, concentrations of radon gas in houses, cystic fibrosis in the United States, infanticide practised by people living at high altitudes, and similar topics of unexplained relevance to food irradiation. When Murray finally comes to his promised subject matter, his presentation is extremely unbalanced.

The section on irradiation of spices should be of particular interest, for example, because in countries where radiation processing is practised on an industrial scale, treatment of spices to improve their hygienic quality constitutes the most important use of this technology. The reader of this book learns nothing of this. Of the 30 or so published research papers on spice irradiation, Murray mentions only two. He claims that irradiated spices lose flavour and that their treatment at the maximum allowed dose fails to reduce the bacterial contamination to a level acceptable to the meat-processing industry. A reference follows, but I found nothing in that source to support the claim.

One chapter deals with radiolytic changes in irradiated foods. Murray's approach can be illustrated by his description of radiation effects on amino acids and proteins. He warns that protein malnutrition will become more widespread should irradiation of cereal grains and legume seeds be permitted. Presumably, the only fact supporting this warning is the 13 per cent loss of tryptophan in beef irradiated at high dose, reported about 25

years ago. The author of the source from which the presumed 13 per cent loss was taken had expressly warned that this was within the experimental error of the method. Murray fails to mention this, and also fails to mention a score of more recent publications which have reported no loss of essential amino acids in any food. He maintains that irradiation of foods will lead to enhanced production of aflatoxins, conveniently overlooking all publications that contradict this claim. He predicts that some of the effects of eating irradiated foods should resemble the effects of vitamin

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Irradiated fruit — headed for an uninformed public?

E deficiency. The reference he cites turns out to be a textbook from 1959 which contains nothing supporting such a prediction.

Many of Murray's firm statements are not supported by data or by references, for instance when he maintains that the

costs involved in irradiating food are prohibitive or that foul flavours are produced, making irradiated food repugnant to the senses. In fact, about 40,000 tons of food were irradiated in Belgium, France and the Netherlands last year. The cost was apparently not prohibitive, and it is difficult to believe that buyers were willing to accept foul-smelling products.

When provided, Murray's references often consist of articles in Australian newspapers, statements presented before committees of the Australian House of Representatives, proceedings of meetings of consumer organizations or similar sources that will not be accessible to most readers. All the chapters are overburdened with irrelevant matter and with references to literature completely unrelated to food irradiation, whereas many of the most relevant studies remain unmentioned. Unwarranted conclusions, misunderstandings and half-truths are mixed with vituperation against those who have defended the use of food irradiation. Murray accuses these people of ignorance, stupidity, misrepresentation and of uttering arrant nonsense. This book cannot even be recommended to those who are opposed to food irradiation. It would provide them with largely unreliable arguments. □

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■ See also the Scientific Correspondence on page 202 of this issue.

Clashing symbols

Stuart Sutherland

Microrecognition: Philosophy, Cognitive Science and Parallel Distributed Processing. By Andy Clark. MIT: 1989. Pp.226. £14.95, \$19.95.

FOR the past few years, artificial intelligence has been split into two camps, to each of which the other is a plague and an abomination. On the one side, there is conventional AI, which attempts to simulate the mind by programs organized in symbols manipulated according to explicit rules. On the other side stand the rebellious connectionists, who construct networks of units which are initially randomly connected together in an excitatory or inhibitory way: there are rules for changing the strengths of the connections according to the patterns of inputs. Such programs have shown a remarkable capacity to learn. If, for example, patterns containing different (but often overlapping) features are repeatedly input, the connectionist machine comes to associate (through excitatory connections) all the

features of each pattern. But a given pattern has no specific symbol — it is represented solely by the excitation of many individual units distributed over the whole network. If a few features of one pattern are now input, all or most of the units active for the whole pattern are activated. Moreover, such programs can be 'taught' what outputs to give by feeding back the correct output for a given input.

Parallel distributed processing (PDP), as such mechanisms are called, has many attractive features. Like the human mind, for example, it is sloppy — it may make mistakes but they are small ones; and damage to some of the units does not cause a calamitous failure since the same input patterns are represented by the activity of many dispersed units. On the other hand, nobody at present knows how to execute on a PDP network a series of consecutive steps, of the sort needed in planning or carrying out a geometrical proof.

Andy Clark has the best of both worlds. He suggests that at lower levels connectionism prevails, whereas at the topmost (conscious) levels there are symbolic structures, implemented on a PDP

architecture. His main reasons are as follows. First, the slow but accurate way in which we make deductions in formal logic or in mathematics suggests serial symbol processing, whereas the flash of insight that tells us a theorem can be proved or that results in a brilliant move by a chess master suggests the instantaneous pattern-matching abilities of PDP. Second, the different parts of the brain carry out different functions, but they must communicate with one another. It would, however, be extremely cumbersome to transfer the whole pattern of connectionist firing from one part to another. Clark suggests that symbols are developed for particular firing patterns and are used to communicate with other parts of the brain, hence alleviating the problem. This is an interesting idea, but could be hard to implement.

Similar views have been expressed by Paul Smolensky, as Clark himself acknowledges: it is a pity Clark does not make clear just how his own ideas differ. Nevertheless, this is an interesting book, perhaps more in its asides than in its con-

clusion. Clark points out that in order to survive it is no good coming up with the right answer if it takes so long that you have already been eaten. There is, as all psychologists but few AI workers know, a trade-off in the mind between speed and accuracy. Perhaps PDP provides the speed, conventional symbol processing the accuracy. Clark castigates the AI fraternity for concentrating on tasks that people are bad at, like chess or scientific theorizing, rather than starting with processes, like vision or emotional states, that evolved much earlier. But maybe he is asking too much: we have to tackle the problems that are currently tractable and it is doubtful if even a billion-dollar research programme would throw much light on self-esteem. *Microrecognition* is a laudable and well argued attempt to bring peace and goodwill to the AI fraternity; if it lowers the self-esteem of both camps, that may be no bad thing. □

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On inspection

Robert G. Webster

The Influenza Viruses. Edited by Robert M. Krug. Plenum: 1989. Pp.434. \$79.50, £59.63.

INFLUENZA viruses continue to outwit us even as more crystallographic information on the structure and function of their spike glycoproteins is compiled and our understanding of their genes and of the molecular basis for their variability and virulence increases. Late last year, in England and Wales, influenza virus A gave us a small glimpse of its potential lethality when the excess deaths from influenza exceeded 5,000. This was not because of a failure in the vaccine-matching game, but because of apathy about taking the available vaccine. Health-care officials given responsibility for raising the public's consciousness should recall that the 1918 "Spanish" influenza killed more people than died in World War I and that the genes encoding the causative viruses are maintained in aquatic birds.

Despite continued outbreaks of influenza throughout the world, research on influenza viruses can be taken as a model for the investigation of pathogens that resist elucidation and prevention. In no other field have so many investigators turned their attention to the task of complete delineation of molecular structure and function. The results have had a huge impact on eukaryotic, molecular and cellular biology and on immunology.

First, both the spike glycoproteins —

the haemagglutinin and neuraminidase — of influenza virus have been crystallized, and the three-dimensional structure of their receptor-binding sites and other functional domains established. This should permit a rational approach to the design of inhibitors aimed at the invariant regions of these proteins and offers an alternative approach to vaccines that have to be re-evaluated annually.

Second, the three-dimensional structure of an antibody-combining site on the neuraminidase has been established and found to comprise five polypeptide loops with about 22 amino acids in direct contact with the antibody. This provides the first detailed information about an antibody epitope on an infectious agent and should form the basis for the future design of more realistic synthetic peptide vaccines.

Third, the available structural information on influenza viruses permits detailed characterization of the potential immune repertoire against strain-specific glycoproteins (mainly haemagglutinin) and cross-reactive conserved internal proteins (mainly nucleoprotein). Such information is critical for understanding the host's defence mechanisms and provides a basis for future attempts to generate immune responses against specific protective epitopes.

Fourth, influenza viruses have also become a model for the study of biosynthesis, processing, transport, maturation and insertion of glycoproteins into cell membranes. Such studies provide a general understanding of the cellular processes involved in protein export from eukaryotic cells.

Fifth, the eight RNA segments of

negative polarity in influenza viruses, each encoding at least one protein, provide the opportunity for genetic reassortment (antigenic shift), which probably contributes to the evolution of human influenza pandemics. (Some of the smaller RNA segments also encode integral membrane proteins.)

Sixth, the unique feature of influenza viral messenger RNA synthesis is 'cap stealing' from newly synthesized host-cell RNA polymerase II transcripts and use of the cell nucleus as the site of synthesis of virus-specific RNAs. The various steps in expression and replication of virus genes could be targets for antiviral agents.

Finally, Von Magnus or defective-interfering particles were first recognized among influenza viruses and have served as a model for the molecular mechanisms of generation of these particles by all viruses. Their origin, mode of action and biological significance *in vivo* remains a puzzle.

Bob Krug has brought together, for the first time, many of the investigators responsible for these advances; their collective knowledge and experience provide the first-hand information needed to convince readers of the 'model' role played by influenza virus research. The negative side of recruiting the most eminent investigators is that some sections in the book contain material that has appeared elsewhere. But the younger associates of the senior investigators have written many of the articles, bringing a fresh viewpoint to well-worked subject matter.

This group of review articles maintains a consistency of quality in its prose and illustrations, and includes the often neglected type B and C viruses. I found the subject index a bit skimpy, but to some extent this distraction was offset by abundant headings and subheadings. Overall, the book has potential appeal to a wide scientific audience, including molecular and cell biologists as well as influenza aficionados. It is a useful addition to Frankel Conrat and Robert Wagner's series *The Viruses* (Plenum, 1989).

If I had to find some small bones to pick, they would be with the absence of a consideration of vaccines and B cell responses; the book took longer than is optimal in going to press and some chapters were better updated after submission than others. A bigger bone would be that there is no concluding overview of why influenza viruses continue to outwit us and what approaches model systems can provide to control influenza. This information is considered in some chapters, but I believe that a general conclusion would have been valuable. □

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The large-scale structure of convection in the Earth's mantle

Peter Olson, Paul G. Silver & Richard W. Carlson

The Earth's mantle is undergoing subsolidus thermal convection, driven by internal radiogenic heat sources and heat conducted from the outer core. Seismic tomographic images reveal a large-scale pattern of convection in the upper mantle compatible with surface plate motions, demonstrating the central role of mantle convection in tectonics. A circum-Pacific ring of descending flow extends throughout the lower mantle. The most controversial issue is the amount of mass exchanged between the upper and lower mantle.

THERMAL convection in the mantle is the most important dynamical process in the Earth's interior. It is the driving force for sea-floor spreading, and the ultimate cause of ocean basins, continental mountain belts, long-term sea-level fluctuations and worldwide earthquake and volcanic activity. The full significance of mantle convection became apparent with the development of plate tectonics in the 1960s, when it was recognized that nearly rigid lithospheric plates, moving at a few centimetres per year, are the upper thermo-mechanical boundary layer of buoyantly driven viscous convective flow deeper in the mantle¹⁻⁴. Oceanic lithosphere forms from hot upwelling mantle at mid-ocean ridges and cools to a depth of 80-100 km after ~100 Myr of sea-floor spreading. Thermal contraction of the cooling lithosphere produces the topography of the mid-ocean ridges, and makes old oceanic lithosphere negatively buoyant and gravitationally unstable at the Earth's surface. The sinking of oceanic lithosphere at subduction zones applies a torque on the viscous mantle and provides the main driving force for convection, including plate motions. This cycle is the 'energy-containing' mode of mantle convection; it is responsible for about 80% of the heat loss from the Earth's interior⁵.

Plate tectonics demonstrates the existence of convective motions, but the internal structure of mantle convection remains controversial. When plate tectonics was first formulated, the prevailing opinion was that convection is confined to the upper mantle⁶, above the 670-km seismic discontinuity. This notion was reinforced by the observation that the Earth's seismicity terminates at a depth of ~670 km, which was interpreted as evidence that subducted oceanic lithosphere is prevented from sinking into the lower mantle⁷. The model of shallow convection was supported by a widely accepted inference that the lower mantle is intrinsically denser than the upper mantle, owing to a large increase in iron content⁸.

But within the last decade, seismic tomography has demonstrated conclusively that thermal convection also occurs in the lower mantle. Now the debate centres on whether the upper and lower mantle convect as a single layer or whether they convect essentially independently, with a limited exchange of mass. Evidence from such diverse fields as seismology, isotope geochemistry, high-pressure mineral physics and fluid dynamics indicates that the upper and lower mantle are not totally separate, but that there is a change in the mode of convection with depth.

Mantle convection differs from thermal convection in an ordinary viscous fluid in several ways, but certainly the most important differences are due to the presence of the lithospheric plates. Whereas the mantle beneath the plates deforms as a viscous fluid, deformation within the lithosphere occurs primarily by brittle failure and faulting, and is strongly concentrated at plate boundaries. Faulting produces plate-boundary structures such as the shear zones associated with transform faults and subduction zones, neither of which occur in ordinary

viscous flow. Because of its proximity to the lithosphere, convection in the upper mantle is strongly influenced by the rigid-body rotations of plates, whereas in the lower mantle, convection conforms more closely to that in a purely viscous fluid. This fact alone may account for much of the difference in the style of convection in the two regions.

Three-dimensional seismology

Seismic compressional and shear wave velocities, the most precisely determined material properties of the mantle, provide the best means for imaging the structure of mantle convection. Most of the variation in seismic velocity is due, however, to the increase in hydrostatic pressure with depth (see Fig. 1), and is unrelated to the structure of convection. Seismic velocities and density increase most sharply in the transition zone between the upper and lower mantle, because of solid-state phase transformations: from olivine to the spinel structure at 400 km (ref. 9) and from spinel to the perovskite structure at 670 km (refs 10-12). The smooth increase in the lower mantle is again due primarily to hydrostatic compression. There appear to be only two regions that have anomalously low velocity gradients: the low-velocity zone (LVZ) at 50-150 km depth and the so-called D'' layer just above the core-mantle boundary (CMB). These coincide with regions of superadiabatic temperature gradients and are produced by convection. Only a few per cent of the radial density variation shown in Fig. 1 can be attributed to radial variations in composition^{13,14}. Nevertheless, it is of utmost importance to determine if any compositional stratification exists, because density stratification resulting from changes in composition with depth would exert a profound influence on mass transfer through the mantle.

Applications of seismic tomography¹⁵⁻¹⁹ have yielded the first global images of three-dimensional mantle structure, in the form of deviations from the spherically averaged profiles in Fig. 1. Lateral heterogeneity in seismic velocity is mostly the result of lateral temperature variation (higher temperatures mean lower seismic wave velocities). The images from global seismic tomography shown in Fig. 2 may be interpreted in terms of cold downwelling and warm upwelling regions, confirming that large-scale subsolidus convection is ubiquitous in the mantle. Comparison of convection patterns in the upper and lower mantle (Fig. 2a and b respectively) indicate that convection is not composed of a simple pattern that can be traced continuously from the surface to the CMB. Instead, it seems that the major descending currents may be continuous, but the major upwellings are not. The region corresponding to this partial discontinuity is the aforementioned transition zone.

In the upper mantle, large-scale seismic heterogeneity correlates with plate tectonics (see Fig. 2a). Shields and platforms—the old, inactive parts of the continents—overlie high-velocity mantle to depths of 200-300 km. The mid-ocean ridge system is represented by low velocities to depths of ~300 km. These

structures have been long recognized from regional studies and are consistent with the interpretation that heterogeneity is caused by temperature differences: low velocities beneath the global ridge system are due to elevated temperatures and perhaps partial melting; high velocities beneath stable tectonic regions are associated with lower-than-average temperatures and thick lithosphere.

Within the lower mantle, the degree of heterogeneity in seismic velocities is smaller than in the upper mantle, but on the largest scale it has a rather simple pattern. It consists of a ring of high-velocity material which, if projected to the surface, is nearly a great circle enclosing most of the Pacific Basin (Fig. 2b). This structure is radially continuous through most of the lower mantle. It separates two broad columns of low-velocity material, one beneath the African continent and the other beneath the south-central Pacific. The distribution of high seismic velocities in the lower mantle correlates with the downward continuation of subducted slabs in the upper mantle beneath convergent plate boundaries¹⁵ (see Fig. 2b). This association indicates that high-velocity subducted slab material extends below the seismicity cutoff and into the lower mantle, although the limited resolution of the lower-mantle structure (wavelengths of 2,000 km or longer) may permit other interpretations. Importantly, the columnar structures with anomalously low velocity are not located beneath mid-ocean ridges, indicating that the large-scale rising motions in the lower and upper mantle are generally not continuous. But the regions of apparently hot lower mantle underlie anomalously high surface topography²⁰, geoid highs, and the two major clusters of volcanic hotspots²¹. Continuous or not, it is clear that lower-mantle upwellings have an effect at the surface. It remains to be explained why the mid-ocean-ridge system is not more closely related to these features.

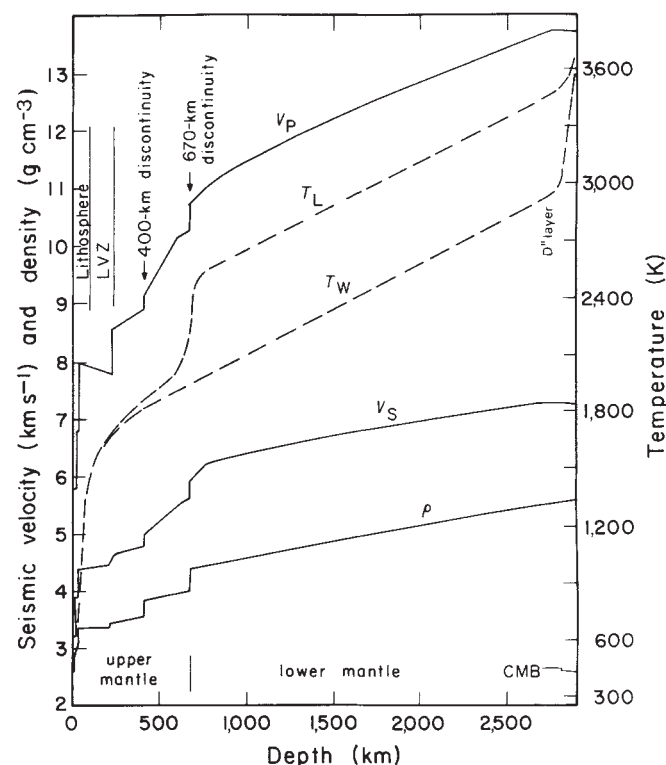


FIG. 1 Profiles of the spherically averaged seismic compressional and shear wave velocities, V_p and V_s , and density ρ through the mantle, from Earth model PREM⁶⁷ (solid lines). Also shown are possible mantle geotherms (dashed lines), including the lithospheric and D''-layer thermal boundary layers. Geotherm T_w is appropriate for whole-mantle convection; T_L is for layered convection; penetrative-convection geotherms are intermediate between these two limits.

Among the most heterogeneous regions in the Earth's interior is the D'' layer, the basal 200–300 km of the mantle. It is notable for a wide spectrum of seismic heterogeneity, ranging from structures that scatter high-frequency body waves²² to 10,000-km-wavelength undulations of the CMB²³. In addition, a laterally variable shear-wave velocity discontinuity located 200–300 km above the CMB is detected over several large regions²⁴. Part of this heterogeneity is no doubt caused by temperature differences, but much of it is probably compositional in origin. The D'' layer may be a repository for former oceanic crust subducted into the lower mantle²⁵. Indeed, it is possible that the core-mantle boundary region is the conjugate of the crust-lithosphere system: a region of strong heat conduction and perhaps chemical differentiation.

The three-dimensional seismic structure of the transition zone has elements in common with both upper and lower mantle structure, and seems to represent a transition in dynamical behaviour as well as in mineralogical properties. The lowest-order heterogeneity consists of a spherical-harmonic degree-two pattern^{16,26} which is closely associated with the present-day locus of subduction. Significantly, neither the global pattern of low velocities in the upper mantle beneath mid-ocean ridges nor the large-scale low-velocity regions of the lower mantle have a clear expression in the transition zone¹⁷.

Direct evidence for continuity between the dynamics of the upper and lower mantle comes from the elastic properties of subducted slabs. Seismic waves propagate 5–10% faster in cold subducted oceanic lithosphere than in the surrounding mantle. Slabs are less than 100 km thick in cross-section, which makes them difficult to detect by global tomographic methods but suitable for regional-scale studies based on travel-time and waveform anomalies from earthquakes located within the slabs themselves^{27–29}. These studies indicate that high-velocity material exists in the lower mantle down-dip of the subducted slabs in the western Pacific, which has been interpreted as the aseismic extension of slabs into the lower mantle²⁷. More recent studies, however, have questioned this interpretation⁷⁷.

In summary, seismic evidence reveals large-scale descending flow in both the upper and lower mantle beneath convergent plate boundaries. Continuous mass flux from the upper mantle into the lower mantle is the simplest interpretation of this pattern³⁰, although other explanations are possible³¹. Major upwellings seem to be discontinuous at the transition zone.

Constraints from mantle-derived isotopes

The pattern of isotopic variations in mantle-derived rocks yields independent information on the character of large-scale convection. Isotopic data from oceanic basalts have provided the most information on this question because of their freedom from the contamination by old, chemically distinct lithosphere that complicates the interpretation of data from continental volcanic rocks. He, Sr, Nd, Hf and Pb isotopic data have revealed that at least four to five isotopically distinct end-members are required to explain the isotopic variation observed in oceanic basalts. The most abundant end-member, a component depleted in incompatible elements (such as potassium, uranium and thorium) relative to the bulk silicate Earth composition, dominates mid-ocean-ridge basalts (MORBs) worldwide. Depletion of the MORB source is not simply a consequence of recent partial melting, but instead reflects melt removal from the mantle over timescales of ~1 Gyr. The strongest candidate for the incompatible-element-enriched complement to the MORB source is the continental crust. Mass-balance calculations involving continental crust and mantle isotopes^{32,33} suggest that about one-third of the mantle, a volume equal to that of the upper mantle, has been depleted of incompatible elements by crust formation. This has led to the interpretation that the MORB source occupies the upper mantle, and overlies a lower mantle composed of relatively undifferentiated material^{34,35}. However, uncertainties in mass-balance calculations based on isotope data

allow the depleted component to range from about 20 to 100% of the mantle^{36,37}.

The remaining isotopically defined components in oceanic volcanism are seen primarily in ocean-island basalts (OIBs) and in MORBs from the Indian Ocean and South Atlantic³⁸. These components are distinguished from normal MORB by containing more radiogenic Sr and Pb, and less radiogenic Nd, which indicates that they are more enriched in incompatible elements than is the normal MORB source. One of these components may be undifferentiated mantle. The elevated $^3\text{He}/^4\text{He}$ ratios found at major hotspots such as Hawaii and Iceland are con-

sistent with a contribution from primitive, undegassed mantle. But this seems to be only a minor component found in a relatively small number of oceanic basalts.

Isotopic model ages for MORB and OIB cluster in the range 1.5–2 Gyr. This striking feature of the oceanic basalt data set is most simply explained as representing the recycling or residence time of oceanic crust, the average time interval between creation of a parcel of oceanic crust and its reappearance as a component of new ocean-ridge volcanism. A recycling time of less than 2 Gyr has important implications for the structure of mantle convection. The present rate of subduction of oceanic litho-

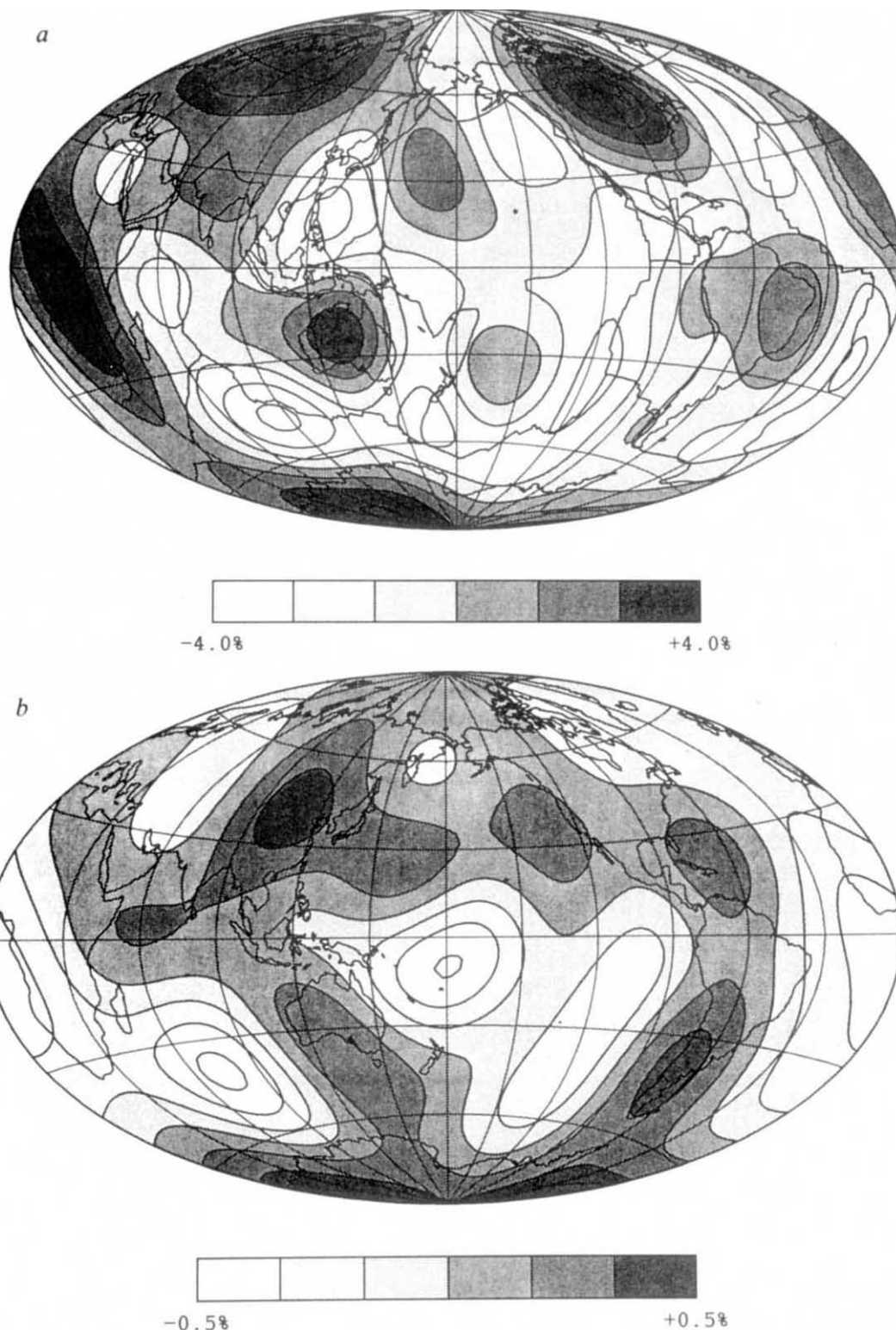


FIG. 2 *a*, Global perturbations in upper-mantle shear wave velocity (relative to model PREM⁶⁷) at 150 km depth, showing the coupling of upper-mantle convection to plate motions. Continental margins and plate boundaries are shown. (From ref. 17.) *b*, Global perturbations in lower-mantle P-wave velocity (relative to model PREM) at 2,200 km depth, showing the circum-Pacific high-velocity ring and the two circular regions of relatively low velocity beneath Africa and south-central Pacific. From ref. 19.

sphere is $3 \text{ km}^2 \text{ yr}^{-1}$ (ref. 39). Assuming that the depth to which partial melting and differentiation occurs extends to 30–75 km (ref. 40), the mean residence time for the whole mantle is in the range 5–14 Gyr, significantly longer than the recycling time inferred from mantle isotopes. To reconcile this difference, it is necessary to postulate either that the rate of plate creation and subduction was much greater in the past, or that only a fraction (roughly one-third) of the mantle is involved in remixing of former lithosphere. The fact that this is nearly the mass fraction of the upper mantle suggests that the reservoir supplying material to the tectonic plates is the upper mantle, not the whole mantle. This configuration is most easily accommodated by 'two-layer' mantle convection, in which the upper and lower mantle are convecting separately⁴¹. It is important to emphasize, however, that the two-layer model is not the only way to resolve the discrepancy between the isotopically inferred recycling time and the residence time based on the whole mantle. An alternative explanation is that the three-dimensional structure of convective motions controls the volume of mantle involved in the remixing process, by preferentially recirculating subducted lithosphere back to spreading centres with a 2-Gyr time constant, leaving most of the mantle unsampled⁴². If, for example, convection in the mantle consists of a steady-state flow pattern with horizontal scales and velocities corresponding to those of the major plates, then recirculation from subduction zone to ridge, through the whole mantle, requires 300–400 Myr. This represents an estimate of the lower bound; all of the complicating effects that we know to be present in mantle convection act to increase the actual recirculation time. The two most important complications are time dependence in the flow pattern^{43–45} and the reduction in convective velocities in the lower mantle owing to a probable increase in viscosity⁴⁵. By invoking these effects, it is easy to increase the recirculation time to 2 Gyr without restricting flow to the upper mantle.

Global-scale lateral variations in mantle-derived isotopes are potentially a more direct constraint on the structure of mantle convection. So far, only a few systematic large-scale trends have been demonstrated conclusively. Perhaps the best documented global heterogeneity is the so-called Dupal anomaly in the South Atlantic and Indian Oceans, where the basalts exhibit elevated $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{207}\text{Pb}/^{204}\text{Pb}$ ratios compared with other oceans⁴⁷. A similar although less distinct anomaly has been identified in the south central Pacific⁴⁸. These large-scale geochemical anomalies coincide with the centres of regions of anomalously low seismic velocity in the lower mantle, with hotspot clusters and relative highs in the geoid⁴⁹, and with anomalous topography²⁰, all indicative of ascending flow.

The geotherm and convective energy sources

The geotherm—the variation of temperature with depth—is a direct result of mantle convection. Temperature profiles in fluids undergoing vigorous convection consist of thin thermal boundary layers with steep temperature gradients, in which heat conduction and heat advection are in balance, bounding interior regions in which heat advection is dominant and the temperature distribution is nearly isentropic⁵⁰. This is an excellent model for the mantle geotherm (Fig. 1). The lithosphere is the surface thermal boundary layer, and the D'' layer includes the basal thermal boundary layer for heat conduction from the core. Most of the rest of the mantle is approximately isentropic, as is the case for fully developed convection. It has not been demonstrated, however, that the upper and lower mantle geotherms lie on the same adiabat, which is a prediction of the whole-mantle convection model. Uncertainties in transition-zone temperatures leave open the possibility of a superadiabatic thermal layer near 670 km (ref. 51), as predicted by the layered convection model.

Important new information on the geotherm deep in the Earth comes from recent high-pressure melting-point determinations of the compounds Fe and FeS thought to be present in the core^{52,53}, and mantle compounds such as silicate perovskite⁵⁴.

Solid mantle and liquid core material must coexist at the CMB, and the boundary between inner and outer core must correspond to the liquidus of the core material. It seems that the adiabat in the outer core is significantly above the adiabat through most of the lower mantle (Fig. 1). In order to join the core and lower-mantle geotherms, a strongly superadiabatic thermal boundary layer must exist at the base of the mantle. Heat conducted across the CMB provides an energy source for initiating thermochemical plumes^{55,56}, which are thought to be the cause of volcanic hotspots.

Modelling mantle convection

Numerical models have proved to be useful for interpreting geophysical and geochemical observations in terms of mantle convection processes. Two different approaches to modelling are in common use; each has been successful in explaining some of the observations.

One approach is to derive the pattern of flow in the mantle from the tomographic images, and to determine whether this flow pattern is capable of explaining surface observables. In this method, the tomographic images are converted to density anomalies and, together with the excess density in the slabs, are entered as body forces into the equation of motion for buoyantly driven viscous flow, thus deriving the instantaneous motions of plates, the geoid and the surface topography. Using this method, Hager and coworkers^{57,58} have shown that the observed positive geoid over the western Pacific subduction zones can be explained in terms of negatively buoyant lithospheric slabs being supported by a 30-fold increase in viscosity between the upper and lower mantle. In addition, much of the remaining long-wavelength geoid is due to lower-mantle density heterogeneity, an important finding which connects seismic velocity heterogeneity, density variations and the geoid. More recent applications of this approach, however, indicate that the body force from slabs and upper-mantle heterogeneity, not lower-mantle heterogeneity, drive plate motions⁵⁹. This another indication of the partially independent nature of the circulations in the two regions.

The ultimate challenge for dynamic models is to demonstrate that the observed pattern of mantle heterogeneity can be explained directly in terms of the theory of thermal convection, and to establish the relationship between the structures in tomographic images and the energy sources that drive mantle convection. Important progress in this direction has been made recently using three-dimensional computations of thermal convection in viscous spherical shells^{60–62}. The large-scale structure of mantle convection as inferred from lower-mantle tomography—a network of descending sheets surrounding a few prominent columnar upwellings—is also the basic structure found in the three-dimensional numerical simulations. The difference between rising and descending motions is due to the combined effects of internal heat production and sphericity.

In fully developed, high-Rayleigh-number thermal convection, the flow is driven by rising and sinking sheets or plumes of buoyant fluid which have separated from the horizontal thermal boundary layers. Convection in a constant-viscosity fluid layer heated entirely from below is symmetrical, in the sense that upwellings and downwellings are statistically equal in both strength and structure. But heat production within the fluid destroys this symmetry by raising the average fluid temperature above the mean temperature of the boundaries. The surface thermal boundary layer is enhanced relative to the basal layer, and the torques driving the motion are concentrated where the surface layer sinks into the fluid. Three-dimensional convection patterns in fluids with internal heat sources consist of an irregular, time-variable network of negatively buoyant, descending sheets of fluid separated by nearly passive upwellings⁶³. This explains why in the mantle, where internal heat generation is an important energy source, plate motions are driven primarily by subducting lithosphere.

In a spherical shell, the asymmetry between rising and sinking

motions occurs even when there is only basal heating. Cylindrical upwellings form above the convex inner boundary, and sheet-like downwellings form below the concave outer boundary. This is illustrated in Fig. 3, which shows the results of calculations of thermal convection in a spherical shell by Bercovici *et al.*⁶¹, in which the flow is driven by basal heating only (Fig. 3a) or by roughly equal amounts of basal and internal heat generation (Fig. 3b). The combination of rising columnar plumes and sinking tabular sheets is evidently a fundamental property of convection in a viscous spherical shell in which there is some basal and some internal heating.

This result is most important for interpreting the structure of thermal convection in the mantle. The combination of ascending columnar plumes and descending sheet structures is similar to the large-scale three-dimensional seismic velocity heterogeneity in the lower mantle. The spherical-shell calculations provide strong evidence that mantle convection is indeed being imaged by seismic tomography (compare the patterns in Figs 2b and 3a). This agreement between calculated and observed patterns also suggests that the structure of convection in the lower mantle can be simulated using relatively simple models of viscous fluids in spherical geometry. However, the Rayleigh numbers of these calculations are only 10^5 , whereas the mantle value is closer to 10^7 (ref. 50). More complex flows, with motion occurring over a wider range of scales, are expected in the real mantle.

Mantle convection with stratification

The development of sophisticated computer models has not provided a definite answer to the question of stratification in

mantle convection. Arguments for stratification come from the need for distinct, long-lasting geochemical reservoirs, as well as evidence for iron enrichment in the lower mantle. The petrological question is whether the transition zone represents only isochemical phase transformations or whether it also marks a change in the bulk composition. The latter alternative necessarily implies restricted mass transfer between convecting systems in the upper and the lower mantle; the former is more consistent with whole-mantle convection.

Transformation of magnesium-rich olivine, $(\text{Mg}, \text{Fe})_2\text{SiO}_4$, the most abundant mineral in the upper mantle, from the spinel structure to a mixture of magnesiowustite $(\text{Mg}, \text{Fe})\text{O}$ and MgSiO_3 in the perovskite structure is found experimentally to occur at approximately the pressure that corresponds to the jump in density and seismic velocity at 670 km (refs 11, 12). Dynamical calculations indicate that isochemical phase transformations in the transition zone by themselves are unlikely to prevent mixing of the upper and lower mantle by convection^{64,65}. A more plausible way to isolate the upper from the lower mantle is by an increase in density owing to a change in bulk composition. As the density of mantle compounds is highly sensitive to iron content, the issue of mantle stratification has most often been cast in terms of whether there exists an increase in iron content with depth.

Jeanloz and Knittle⁶⁶ have recently subjected representative upper-mantle compositions to the pressures and temperatures of the lower mantle, and have compared their density with the lower-mantle density obtained from Earth model PREM.⁶⁷ The comparison involves a large correction for the higher

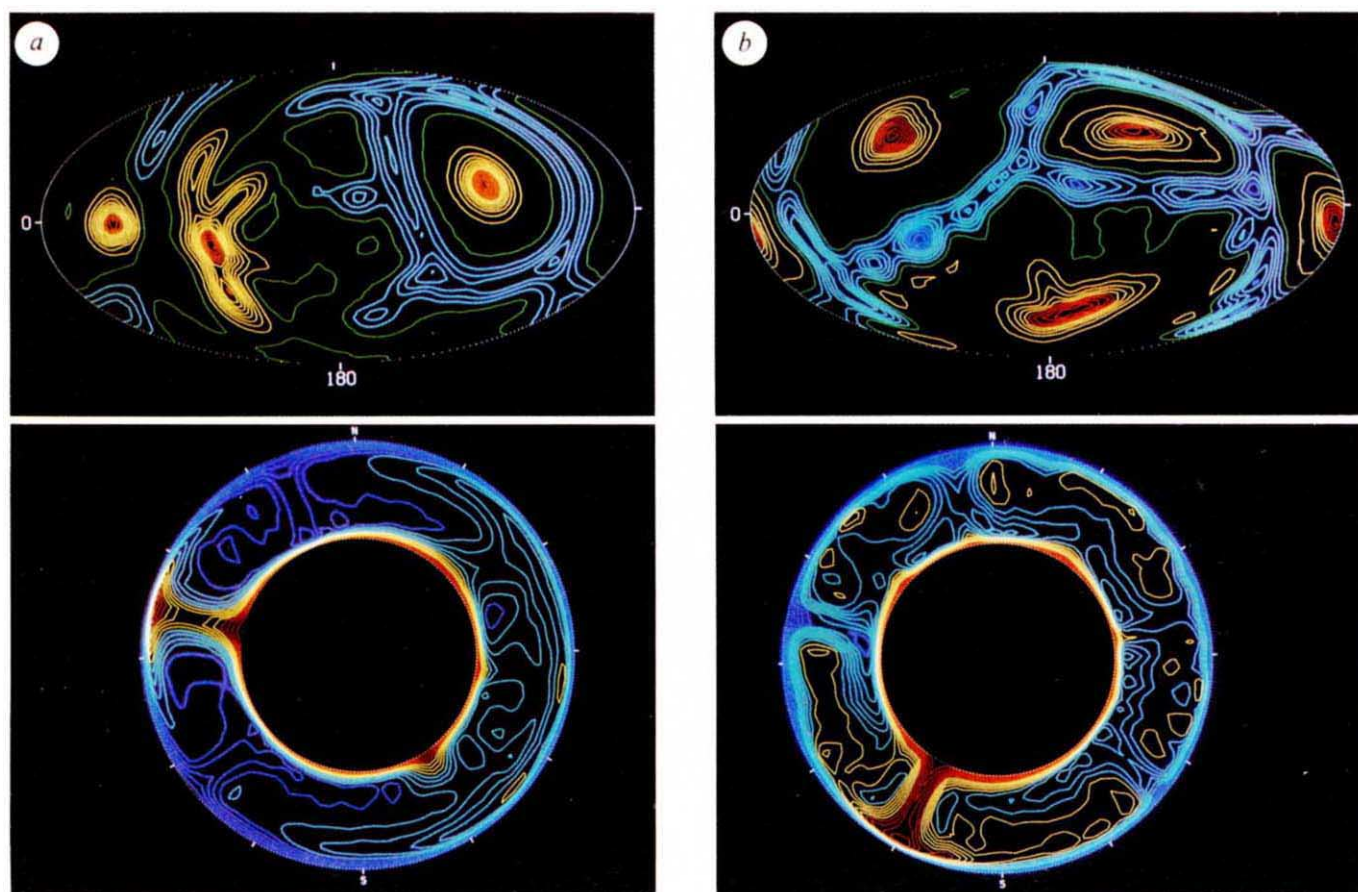


FIG. 3 Numerical calculations⁶¹ of three-dimensional thermal convection in a spherical shell of an infinite-Prandtl-number (highly viscous), compressible fluid. *a*, Base-heated convection at Rayleigh number $Ra \approx 10^5$. (The Rayleigh number is essentially a measure of the ratio of temperature differential to fluid viscosity.) The top panel shows contours of vertical velocity at mid-depth. Velocity contours are colour-coded from red (maximum) to blue

(minimum). The bottom panel shows entropy (potential temperature) contours in one great circle, colour-coded from red (hot) to blue (cold). *b*, Convection with equal amounts of internal and base heating. Top and bottom panels show the same quantities as in *a*. Note the similarity between the shape of up- and downwellings in these simulations and those in the lower-mantle tomographic image in Fig. 2b. Photographs from ref. 61.

temperatures in the lower mantle, because measurements indicate that the perovskite phase has a (large) thermal expansion coefficient in the range $3\text{--}4 \times 10^{-5} \text{ K}^{-1}$ at lower-mantle conditions¹⁴. Critical parameters in this comparison are the iron content of the upper mantle and the temperature in the lower mantle. Iron content is expressed indirectly in terms of the magnesium number $X_{\text{Mg}} = 100 \times \text{Mg}/(\text{Mg} + \text{Fe})$. Estimates for the upper mantle give $X_{\text{Mg}} \approx 89$. This composition matches PREM lower-mantle densities only if lower-mantle temperatures are quite low, 1,500 K or less⁶⁶. A more likely lower-mantle temperature, in excess of 2,000 K, implies that the composition with $X_{\text{Mg}} = 89$ is intrinsically less dense than the lower mantle by about $4 \pm 2\%$. According to this comparison, the upper and lower mantle differ in bulk composition and therefore have not been fully homogenized by convection.

Weak compositional stratification may prove to be a satisfactory model for the petrology of the mantle, but it raises several interesting geodynamical problems. A major question is the stability of such layering in the presence of thermal convection. It has been demonstrated both experimentally^{69,70,72} and by calculations^{64,71} that the stability of layered convection depends simply on a global parameter R_ρ , the ratio of the total compositional density variation to the density variation from superadiabatic temperature gradients. Overturn occurs when $R_\rho < 1$; strictly layered convection occurs for $R_\rho \gg 1$. In the critical neighbourhood of $R_\rho \approx 1$, penetrative convection occurs, in which the layers mix slowly by entrainment and exchange processes, and the boundary between the two layers is highly distorted by the flow. In which dynamical regime does the mantle lie? The large thermal expansivity for perovskite implies, in addition to iron enrichment, a large thermal buoyancy in the lower mantle. Using the geotherms in Fig. 1, the superadiabatic thermal density difference across the whole mantle is $\sim 4\text{--}6\%$, indicating that $R_\rho \approx 1$ for the stratified mantle model, which is dynamically inconsistent with the existence of strictly horizontal layers. However, $R_\rho \approx 1$ is consistent with penetrative convection, in which mechanically strong subducted slabs from the iron-depleted upper mantle sink transiently into the lower mantle without causing massive overturn^{30,64,71,72}.

Even without chemical layering, a statistical stratification in the age of mantle rocks can result from a change in the pattern of convection owing to an increase in viscosity with depth⁴⁶. The reservoir effect recorded in mantle isotopes may simply be the result of physical separation of the material entering the more viscous lower mantle, where the residence time is several thousand million years, from the material remaining in the upper mantle, where the convection is more vigorous and the residence time is shorter.

Effect of the continents

The form of compositional heterogeneity whose dynamical behaviour is best understood is that of the continental crust. Interaction between continents and mantle convection is governed by the fact that continents are buoyant in the upper mantle and resist subduction. The history of large-scale continental tectonics consists of aggregation into supercontinents and subsequent dispersal. The present position of large-scale geophysical anomalies, relative to both the present drift direction of the continents and the reconstructed site of the most recent supercontinent Pangea, suggests how this cycle works. About 200 Myr ago, Pangea was located at the present position of the African–Atlantic geoid high⁷³. This geoid high is itself located above the largest hotspot cluster and above one of the major regions of anomalously low seismic velocity in the lower mantle, indicating that it marks a lower-mantle upwelling. Since the breakup, Australia, Antarctica, India and the Americas have all been drifting toward lows in the geoid. These patterns suggest that supercontinental breakup is associated with major lower-mantle upwellings, and conversely, that the dispersed state involves the drift of continents toward the sites of major down-

wellings⁷⁴. Recent simulations of this process using numerical models explain this behaviour in terms of continental masses interacting dynamically with mantle convection⁷⁵. Continents drift toward subduction zones, collisions occur, and downwelling beneath the supercontinent ceases. New subduction zones form near the margins of the supercontinent, and induce upwelling beneath the supercontinental interior, leading to breakup. According to this model, large blocks of continental crust on the Earth's surface induce a long-term cycle in the pattern of mantle convection, with periods of supercontinent formation separated by episodes of continental drift.

Future directions

Several important developments are anticipated in the near future that should further resolve the structure of mantle convection. The most important issue is to determine the fate of oceanic lithosphere, which could be resolved by seismic experiments that image the trajectory of subducted slabs beyond the seismicity cutoff. Progress on this question has been made already but further advances will rely more on regional-scale tomographic experiments using large numbers of high-quality instruments to resolve the deep structure beneath subduction zones on scales of 100 km. Similarly conceived experiments can be made around hotspots such as Hawaii to prospect for the deep structure of mantle plumes, and on ridges to determine the depth of upwelling beneath spreading centres.

Rapid advances in computational hardware and software make it likely that realistic numerical simulations of mantle convection—simulations using three-dimensional spherical geometry, subsolidus rheology, and parameter values appropriate for the mantle—can be made in the near future. The most significant shortcoming of present-day models is their treatment of lithospheric plates. Simplified schemes for incorporating the effect of plates are already in use^{5,64,76}, but further development is required. To simulate lithospheric plates correctly in global-scale convection models, it is necessary to incorporate the smaller-scale processes of plate-boundary deformation, such as transform and subduction zone faulting, rifting and partial-melt transport at spreading centres. □

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1. Turcotte, D. L. & Oxburgh, E. R. *J. Fluid Mech.* **28**, 29–42 (1967).
2. Elsasser, W. M. in *The Application of Modern Physics to the Earth and Planetary Interiors* (ed. Runcorn, S. K.) 223–246 (Wiley, New York, 1969).
3. McKenzie, D. P. *Geophys. J. R. astr. Soc.* **1**, 1–32 (1969).
4. Richter, F. M. *Rev. Geophys. Space Phys.* **2**, 223–287 (1973).
5. Davies, G. F. *J. geophys. Res.* **93**, 10451–10466 (1988).
6. McKenzie, D. P., Roberts, J. M. & Weiss, N. O. *J. Fluid Mech.* **62**, 465–538 (1974).
7. Richter, F. M. *J. geophys. Res.* **84**, 6783–6795 (1979).
8. Anderson, D. L. *Miner. Soc. Am. spec. Pap.* **3**, 85–93 (1970).
9. Akaogi, M., Ito, E. & Navrotsky, A. *J. geophys. Res.* **94**, 15671–15685 (1989).
10. Liu, L. G. *Earth planet. Sci. Lett.* **62**, 91–103 (1979).
11. Ito, E. & Yamada, H. in *High Pressure Research in Geophysics* (eds Akimoto, S. & Manghnani, M. H.) 405–419 (Tokyo Centre. Acad. Publ., 1982).
12. Ito, E. & Takahashi, E. *J. geophys. Res.* **94**, 10637–10646 (1989).
13. Weidner, D. J. & Ito, E. in *High Pressure Research in Mineral Physics* 117–124 (Am. Geophys. Union, Washington DC, 1987).
14. Knittle, E., Jeanloz, R. & Smith, G. L. *Nature* **319**, 214–216 (1986).
15. Dziewonski, A. M. *J. geophys. Res.* **89**, 5929–5952 (1984).
16. Woodhouse, J. H. & Dziewonski, A. M. *J. geophys. Res.* **89**, 5953–5986 (1984).
17. Dziewonski, A. M. & Woodhouse, J. H. *Science* **236**, 37–48 (1987).
18. Dziewonski, A. M., Hager, B. H. & O'Connell, R. J. *J. geophys. Res.* **82**, 239–255 (1977).
19. Giardini, D., Li, X.-D. & Woodhouse, J. A. *Nature* **325**, 405 (1987).
20. Cazenave, A., Souriau, A. & Dominh, K. *Nature* **340**, 54–47 (1989).
21. Richards, M. A., Hager, B. H., & Sleep, N. A. *J. geophys. Res.* **93**, 7690–7708 (1988).
22. Haddon, R. A. W. *Phil. Trans. R. Soc. A* **306**, 61–70 (1982).
23. Morelli, A. & Dziewonski, A. M. *Nature* **325**, 678–683 (1987).
24. Young, C. J. & Lay, T. A. *Rev. Earth planet. Sci.* **15**, 25–46 (1987).
25. Hofmann, A. W. & White, W. M. *Earth planet. Sci. Lett.* **57**, 421–436 (1982).
26. Masters, G., Jordan, T., Silver, P. G. & Gilbert, F. *Nature* **298**, 609–613 (1982).
27. Creager, K. C. & Jordan, T. *J. geophys. Res.* **89**, 3031–3049 (1984).
28. Creager, K. C. & Jordan, T. *J. geophys. Res.* **91**, 3573–3589 (1986).
29. Silver, P. G. & Chan, W. W. *J. geophys. Res.* **91**, 13787–13802 (1986).
30. Silver, P. G., Carlson, R. W. & Olson, P. A. *Rev. Earth planet. Sci.* **16**, 477–541 (1987).

31. Anderson, D. L. *J. geophys. Res.* **92**, 13968–13980 (1987).
32. O'Nions, R. K., Evensen, N. M. & Hamilton, P. J. *J. geophys. Res.* **84**, 6091–6101 (1979).
33. Jacobsen, S. & Wasserburg, G. J. *J. geophys. Res.* **84**, 7411–7427 (1979).
34. Wasserburg, G. J. & DePaolo, D. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3594–3598 (1979).
35. Allègre, C. J. & Turcotte, D. L. *Nature* **323**, 123–127 (1986).
36. Allègre, C. J., Hart, S. R. & Minster, J. F. *Earth planet. Sci. Lett.* **66**, 177–190 (1983); **66**, 191–213 (1983).
37. Carlson, R. W. *Geochem. cosmochim. Acta* **48**, 2357–2372 (1984).
38. Zindler, A. & Hart, S. R. *Rev. Earth planet. Sci.* **14**, 483–571 (1986).
39. Parsons, B. *Geophys. J. R. astr. Soc.* **67**, 437–448 (1981).
40. Basaltic Volcanism Study Project *Basaltic Volcanism on the Terrestrial Planets* (Pergamon, New York, 1981).
41. Jeanloz, R. & Richter, F. M. *J. geophys. Res.* **84**, 5497–5504 (1979).
42. Spohn, T. & Schubert, G. *J. geophys. Res.* **87**, 4682–4696 (1982).
43. Jarvis, G. T. *Phys. Earth planet. Inter.* **36**, 305–327 (1984).
44. Christensen, U. *Geophys. Res. Lett.* **14**, 220–223 (1987).
45. Machetel, P. & Yuen, D. A. *Earth planet. Sci. Lett.* **86**, 93–104 (1987).
46. Gurnis, M. & Davies, G. F. *J. geophys. Res.* **91**, 6375–6395 (1986).
47. Dupre, B. & Allègre, C. J. *Nature* **303**, 142–146 (1983).
48. Hart, S. R. *Nature* **309**, 753–757 (1984).
49. Castillo, P. *Nature* **336**, 667–670 (1988).
50. Jarvis, G. T. & Peltier, W. R. *Geophys. J. R. astr. Soc.* **68**, 389–427 (1982).
51. Jeanloz, R. & Morris, S. A. *Rev. Earth planet. Sci.* **14**, 377–415 (1986).
52. Brown, J. M., Ahrens, T. J. & Sharpine, D. L. *J. geophys. Res.* **89**, 6041–6048 (1984).
53. Williams, Q., Jeanloz, R., Bass, J. & Ahrens, T. J. *Science* **236**, 181–182 (1987).
54. Heinz, D. L. & Jeanloz, R. *J. geophys. Res.* **92**, 11437–11444 (1987).
55. Olson, P., Schubert, G. & Anderson, C. *Nature* **327**, 409–413 (1987).
56. Hansen, U. & Yuen, D. A. *Nature* **334**, 237–240 (1988).
57. Hager, B. H. *J. geophys. Res.* **89**, 6003–6015 (1984).
58. Hager, B. H., Clayton, R. W., Richards, M. A., Comer, R. P. & Dziewonski, A. M. *Nature* **313**, 541–545 (1985).
59. Ricard, Y. & Vigny, C. *J. geophys. Res.* **94**, 17543–17560 (1989).
60. Glatzmaier, G. A. *Geophys. Astrophys. Fluid Dyn.* **43**, 223–264 (1989).
61. Bercowski, D., Schubert, G. & Glatzmaier, G. A. *Science* **244**, 950–955 (1989).
62. Bercowski, D., Schubert, G. & Glatzmaier, G. A. *Geophys. Res. Lett.* **16**, 617–621 (1989).
63. Houseman, G. *Nature* **332**, 346–349 (1988).
64. Christensen, U. & Yuen, D. A. *J. geophys. Res.* **89**, 4389–4402 (1984).
65. Christensen, U. *Phil. Trans. R. Soc. Lond. A* **328**, 417–424 (1989).
66. Jeanloz, R. & Knittle, E. *Phil. Trans. R. Soc. Lond. A* **328**, 377–389 (1989).
67. Dziewonski, A. M. & Anderson, D. A. *Phys. Earth planet. Inter.* **25**, 297–356 (1981).
68. Ringwood, A. E. *Composition and Petrology of the Earth's Mantle* (McGraw-Hill, New York, 1975).
69. Richter, F. M. & McKenzie, D. P. *J. geophys. Res.* **86**, 6133–6124 (1981).
70. Olson, P. *J. geophys. Res.* **89**, 11293–11301 (1984).
71. Ellsworth, K. & Schubert, G. *Geophys. J.* **93**, 347–363 (1988).
72. Kincaid, C. & Olson, P. *J. geophys. Res.* **92**, 13832–13840 (1987).
73. Chase, C. G. *Nature* **292**, 464–468 (1979).
74. Anderson, D. L. *Nature* **297**, 391–393 (1982).
75. Gurnis, M. *Nature* **332**, 695–699 (1988).
76. Gurnis, M. & Hager, B. H. *Nature* **335**, 317–321 (1988).
77. Zhou, H. & Anderson, D. L. *Proc. natn. Acad. Sci. USA* **86**, 8602–8606 (1989).

Unusual composition of pore waters found in the Izu–Bonin fore-arc sedimentary basin

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Extensively altered brines, with a composition different from that of any previously reported natural water, have been recovered by deep coring of sediments in the Izu–Bonin fore-arc sedimentary basin. Samples from successive depths document the chemical evolution of these unusual pore waters, from sea water to highly enriched CaCl_2 brines. The origin of these pore waters seems to be related to the special environment of a fore-arc sedimentary basin, where accumulation rates of volcanogenic sediment are extremely high and the sediments are susceptible to alteration.

MOST processes that lead to extreme composition of natural waters involve large shifts in the total concentration of salt (for example, evaporation, reverse osmosis and dissolution of evaporites). Here we show how purely chemical reactions between water and minerals may give rise to highly altered waters without concomitant large changes in the concentration of total dissolved solids.

Brines rich in CaCl_2 are an exotic class of natural waters whose genesis has been much debated. They have been found in a variety of situations, including some oil-field formation waters¹, as fluid inclusions in ore minerals², in some saline lakes (such as the Dead Sea³), in modern rift-zone hydrothermal brines⁴ and as ground waters in crystalline rocks⁵. Several

mechanisms have been suggested for their formation, including shale membrane filtration¹, interaction with evaporites⁶, dolomitization⁷, reactions of sea water with basalt⁴ and the reactions of metamorphic HCl with carbonates⁸. We now describe the finding of a distinctive CaCl_2 brine in a different geological setting whose mode of formation is novel.

The setting is the Izu–Bonin fore-arc sedimentary basin south-west of Japan (Fig. 1), in the western half (site 792) and the centre (site 793) of which two holes were drilled on Leg 126 of the Ocean Drilling Program. The brines occurring as pore waters at the two sites are characterized by very large Ca/Na ratios and comparatively small amounts of total dissolved solids (TDS). They originated as sea water and are almost completely depleted in Mg and K, and the concentration of SO_4 is reduced by 50%. The pore water is also rich in Cl, its alkalinity and silica content are low and the pH is high.

These distinctive features of the Izu–Bonin pore water are explained by the special environment in which they occur. At both sites, volcanogenic sediments that are highly susceptible to chemical alteration have accumulated rapidly, the temperature is moderately high (<80 °C) and diffusivities are very low. At one of the sites, the governing reactions—low-temperature alteration of volcanogenic sediments into zeolites, smectites, carbonates and gypsum—have been sufficiently extensive to cause marked uptake of water into secondary minerals.

Geological setting

Palaeontological and sedimentological data suggest that the fore-arc basin was formed by rifting no earlier than the middle Oligocene, and that the newly formed basin had initial depths

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of 4–5 km. Sedimentation occurred initially at a rate of 250–300 m Myr⁻¹ and debris-flow deposits and turbidites were produced by concurrent volcanism and erosion of surrounding topographic highs. By the early Miocene, arc volcanism had become very much reduced, as evidenced by the slow accumulation (~10 m Myr⁻¹) of claystones and nannofossil claystones. The sediments deposited during the initial volcanic period constitute the interval from 429.3 m below sea floor (m.b.s.f.) to 804 m.b.s.f. at site 792 and the interval from 759 m.b.s.f. to 1403.9 m.b.s.f. at site 793. Here we use a simplified lithostratigraphic scheme consisting of the Oligocene volcanogenic section and the post-Oligocene, mainly detrital and biogenic section. The boundaries between these two sections are indicated in Fig. 2.

At both sites the transition downward from the Miocene to the Oligocene section is distinguished by a sharp decrease in biogenic carbonate and organic carbon followed by an equally sharp increase in the abundance of zeolites, smectites, palagonite and gypsum. Volumetrically less important authigenic minerals are celadonite, dolomite and ankerite. For a more detailed mineralogical and lithological description see ref. 9. Sound velocity and sediment electrical resistivity change most abruptly across the Miocene/Oligocene boundary. The sound velocities increase by 0.5–1.0 km s⁻¹, and the sediment electrical resistivities increase from an average of ~0.9 Ω m to ~3.0 and 4.3 Ω m in the Oligocene sections at site 792 and 793 respectively (corrected to 25 °C). The corresponding formation factors in the Oligocene sections (averaging 12 and 22 at site 792 and site 793 respectively) are exceptionally high for the given porosities (40–70% at site 792, 20–60% at site 793).

Water chemistry

The methods used for sampling and analysis have been described elsewhere⁹. The distribution of the major cations (Fig. 2a, b) follows the pattern commonly found for marine sediments¹⁰: decreasing concentrations of Mg, K and Na and increasing Ca. But the extent of Ca increase and Na decline in the Oligocene sections is more pronounced than any reported previously for deep-sea sediments. Aside from evaporitic environments, the highest Ca concentration reported previously is 125 mM, which

was in a sample from a sediment-sill complex¹¹. At site 792, a maximum Ca concentration of 169.3 mM has been observed at 599 m.b.s.f., and at site 793 the concentration of Ca reaches a stable level of ~300 mM below 997 m.b.s.f. The concentration of Na in this region is ~160 mM. The low concentration of Mg (Fig. 2b) shows that the reversal of the concentration profiles of Na and Ca towards the basement at site 792 cannot be the result of contamination by sea water during drilling. At both sites, the concentration of K and Mg drop towards zero across the Miocene/Oligocene boundary (Fig. 2b), and the concentration of SO₄ decreases by ~50% (Fig. 2c). At site 793, a significant increase in Cl is observed across the Miocene/Oligocene boundary and a stable level of ~710 mM is reached below 997 m.b.s.f. (Fig. 2c). Moreover, pore-water titration alkalinities decrease from 2–4 mM to ~0.5 mM in the Oligocene sections, pH increases by about 1 pH unit from 7.7 in the Miocene sections, and there is a pronounced drop in the concentration of dissolved silica (from 680 μ M to 220 μ M) in the Oligocene sections (average for both sites).

Comparison with other CaCl₂-rich brines

To identify brines, we follow the scheme of Krieger¹², who defines them as natural waters containing more than 35,000 p.p.m. of dissolved solids. According to this scheme, all of the pore waters plot on the boundary between very saline waters and brine. White *et al.*¹³ defined calcium-rich natural waters using the arbitrary criterion [Ca] = 0.1[Na]. More recently⁴, CaCl₂-rich waters were defined explicitly as waters in which 2mCa > 2mSO₄ + mHCO₃ + 2mCO₃ (m = molality). This definition is the most useful as it specifically draws attention to the presence of a CaCl₂ component. But because of the uncertainty regarding analyses of sulphate in fluid inclusions and the almost complete lack of HCO₃ analyses, we have, for the purpose of comparison with other CaCl₂-rich brines, plotted log(Ca/Na) against TDS (Fig. 3).

Figure 3 shows that the most evolved pore waters in the Izu-Bonin fore-arc sedimentary basin constitute a distinct type of CaCl₂ brines which are characterized by very high Ca/Na ratios at comparatively low concentrations of TDS. The Ca/Na ratios of pore waters from sites 792 and 793 range from that of sea water (0.022) to 1.77. At low TDS, the composition of the sedimentary CaCl₂ brines (field D) merges with the composition for the least altered pore waters recovered during Leg 126 (field A). The composition of these latter waters evolves so as to increase the differences with the CaCl₂ brines reported here. At high Ca/Na ratios, the field corresponding to ground waters from the Canadian Shield (field C) extends close to the most evolved part of field A. This, however, is probably not the result of chemical evolution, but indicates that these waters are mixtures of meteoric water and a very concentrated brine (TDS > 300 g l⁻¹)⁵. The fluids from Iceland (field B) and the ridge-crest hydrothermal solutions are the only ones that seem to be related to the brines reported here, suggesting that they share a common origin and have parallel evolutionary paths.

Mechanism of formation

The simultaneous high Cl and high Ca concentrations at site 793 may not be coincidental. Processes leading to enhanced Cl concentrations in pore waters include dissolution of evaporites, gas hydrate formation, shale membrane filtration and uptake of water by secondary hydrous minerals. Because of the absence of evaporites and the low concentration of organic carbon (average of ~0.03% in the Oligocene section), the first two of these processes may be excluded in this case. The efficiency of membrane filtration is highly dependent on the state of compaction, and none of the claystones at site 793 has sufficiently low porosity to act as a semi-permeable membrane. Furthermore, if the elevated Cl concentrations were caused by shale membrane filtration, one would expect to observe a corresponding Cl minimum on the low-pressure side (the Miocene). Also, a com-

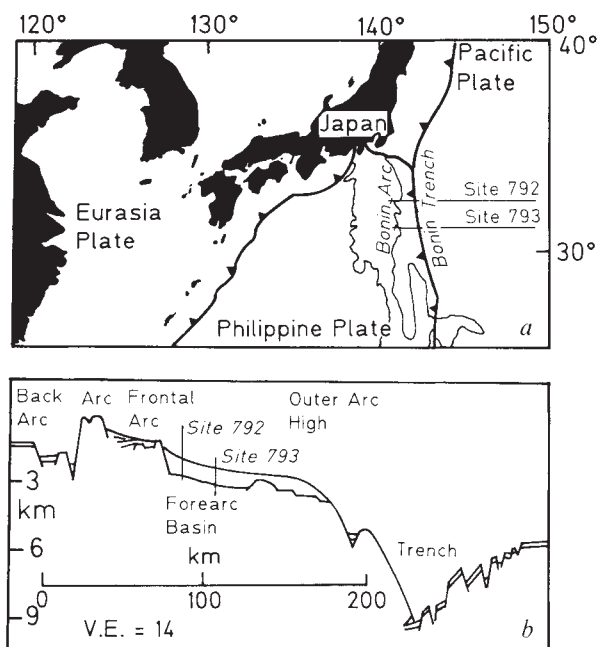


FIG. 1 a, Location of the sites in the Pacific Ocean. b, Projection of the sites onto a schematic section across the Izu-Bonin Arc. At site 792 (793) a total of 804 m (1,682 m) of sediments and 82 m (279 m) of basement rocks was drilled. V.E., vertical exaggeration.

parison with the compositions of the formation waters from the Illinois and Michigan basins (Fig. 3), which are believed to have been generated by this process¹, shows that this mechanism has not been operating here.

We therefore suggest that the high Cl concentrations are caused by uptake of water by secondary minerals. To our knowledge, this is the most extensive loss of water ever reported for pore waters from deep-sea sediments. Where do the governing reactions take place? Several lines of evidence strongly support the idea that these extreme pore-water compositions are caused by reactions taking place in the Oligocene sedimentary section, and are not related to basement processes: first, authigenic zeolites, smectites and gypsum are abundant in the Oligocene sections; second, concentration gradients towards the basement are reversed (site 792) or absent (site 793); third, concentrations of Mg and K drop towards zero at the Miocene/Oligocene boundary (Fig. 2b); and fourth, electrical resistivities of the Oligocene sediments are exceptionally high. The latter argument relies on the fact that the diffusion coefficients of pore-water constituents are proportional to $(PF)^{-1}$, where P is the fractional porosity and F is the formation factor¹⁴. The very low rate of diffusion sustains steep concentration gradients and more pronounced extremes.

The higher diffusivity is probably one reason why the pore waters at site 792 seem to be less altered than those at site 793. The *in situ* temperature is also important: high temperatures lead to greater reaction rates and larger extremes of concentration. Discrete down-hole temperature measurements at site 792 give a thermal gradient of $54^{\circ}\text{C km}^{-1}$. The Oligocene section at site 793 is deeper and so will have a higher temperature and correspondingly higher reaction rates, leading to more-evolved pore waters.

We can make only general statements with regard to which specific reactions might be taking place. Volcanic glass is thermodynamically unstable and decomposes more readily than nearly all associated mineral phases. Reactions involving volcanoclastic material have long been known to influence the composition of pore waters¹⁵, with typical reaction products being zeolites and smectites. The Oligocene section consists of graded beds of vitric sandstones and fine-grained rocks in nearly equal proportions. The proportions of the various framework grains in the sandstones at site 793 are: volcanic glass, 71%; plagioclase, 15%; lithics, 4%; and heavy minerals, 9%. Vitric grains are classed as volcanic glasses, and are largely altered to zeolites and/or green to brownish-green clay minerals. Most of the plagioclases are replaced partially or completely by zeolites.

The average porosity of the Oligocene section at site 793 is 36%. To result in high Cl concentrations, the formation of secondary minerals must have led to an increase of at least 15% in the water content of the solid matrix. Aagaard *et al.*¹⁶ have proposed a set of reactions that lead to the diagenetic transformation of volcanic glass into smectites and zeolites. Although the exact chemistry and mineralogy is probably different here, the water budget will not be much affected. If we assume the same stoichiometry as in ref. 16, the required water uptake can be accomplished by alteration of 25% (by volume) of the rock matrix. This figure is strongly dependent on the porosity: at a porosity of 25%, for example, only 15% (by volume) of the rock matrix need be altered. The method used for determining the porosity (which involves heating the sediments to 105°C for 24 hours) may have led to overestimation because of loss of water absorbed in the zeolites and smectites.

As the Oligocene sections consist of material produced by synrift volcanism and local erosion, we have chosen the composition of unaltered samples from the andesitic basement to

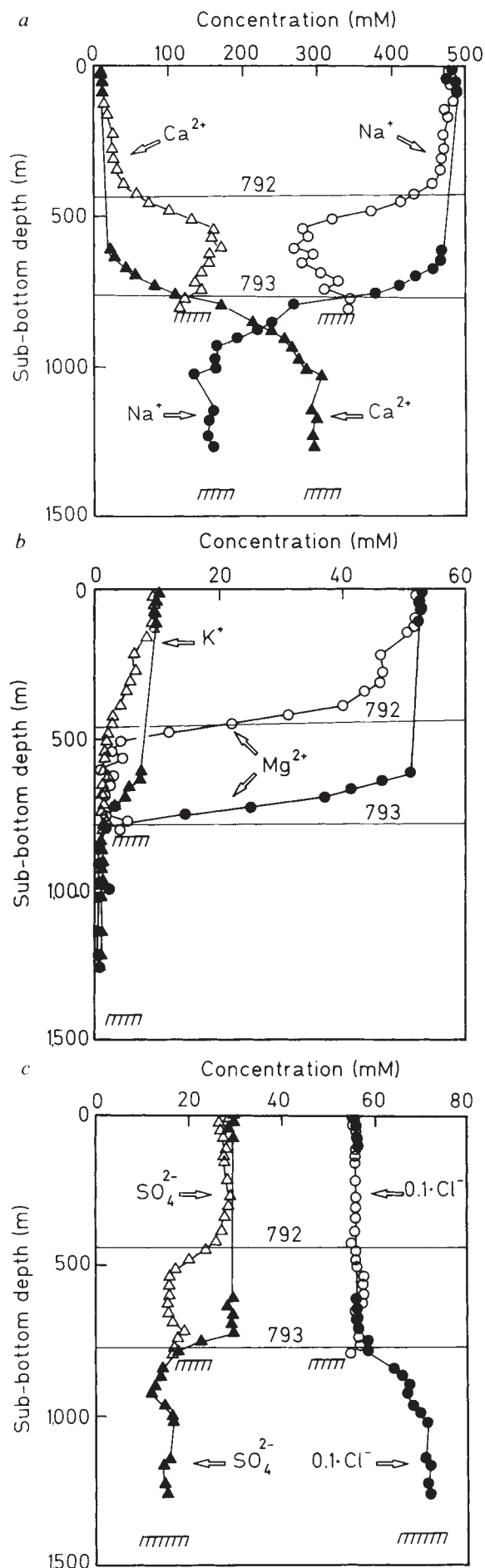


FIG. 2a Concentrations of a, Na and Ca, b, K and Mg, and c, SO_4 and Cl in pore water from site 792 (open symbols) and site 793 (filled symbols). Horizontal lines indicate the position of the Miocene/Oligocene boundary and the depth of basement is indicated by the hatched areas.

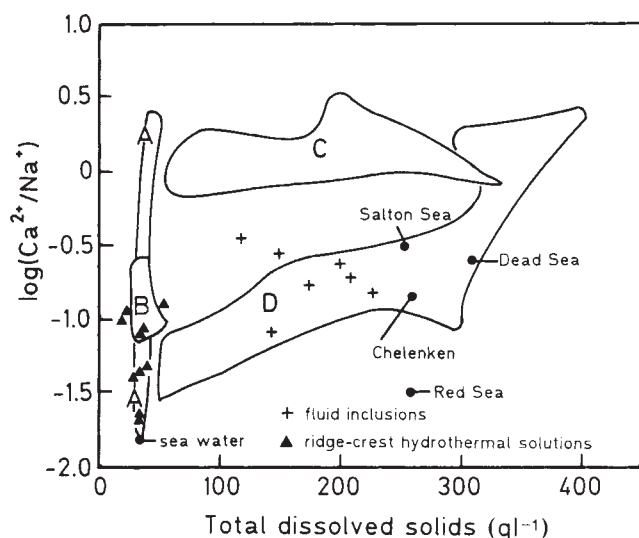


FIG. 3 Ca/Na ratios plotted against total amount of dissolved solids for sites 792 and 793 (field A), hydrothermal waters from Iceland²⁰ (field B), ground waters from the Canadian Shield⁶ (field C), formation waters from the Illinois and Michigan basins¹ (field D), the geothermal fields Chelkenen²¹ and Salton Sea²², the Dead Sea³, the Red Sea²³, fluid inclusions from hydrothermal ore minerals^{24,25} and ridge-crest hydrothermal solutions²⁶⁻²⁸. Only data on waters more concentrated than sea water have been included.

represent the starting point of diagenesis. These samples contain ~10 wt% CaO. Alteration of 25 vol% of the rock matrix would release enough Ca to give a pore-water Ca concentration of more than 2,000 mM. Evidently, most of the Ca must have been incorporated in secondary minerals. This is consistent with our studies of the mineralogy, which revealed the presence of heulandite, stilbite, wairakite, tri-octahedral smectites and gypsum. The tri-octahedral smectites are probably the major sink for Mg and Fe, and analcime, clinoptilolite and phillipsite act as sinks for Na and K.

The evolution of the pore-water composition is in qualitative agreement with results of experimental alteration of basalt by sea water at low temperature (70 °C)¹⁷, but in our case the degree of alteration is much more pronounced. This is probably the result of the higher water/rock ratio of 10 used (ref. 17). Two other interesting discrepancies between our observations and the experimental results may also be related to the water/rock ratio. First, the pH in the experiments dropped from 7.7 to 6.9 and second the concentration of SiO₂ increased to 45.8 p.p.m., whereas at site 793, the pH increases from about 7.6 to 8.9 across the Miocene/Oligocene boundary and the concentration of SiO₂ drops towards quartz saturation (Fig. 4). Removal of Mg from sea water leads to rapidly decreasing pH, which then increases again when the Mg concentration drops to a low level¹⁸. In low-temperature experiments of ref. 17, the concentration of Mg never dropped by more than 13%, so the pH remained low.

Figure 4 shows that with increasing temperature the con-

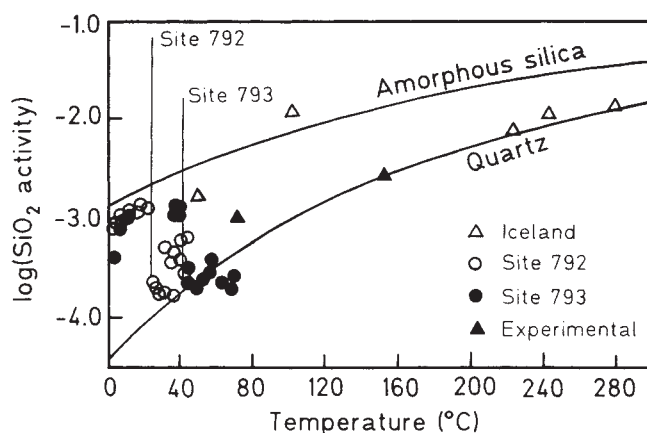


FIG. 4 Activity of SiO₂ (calculated with the geochemical computer code SOLMINEQ²⁹) against *in situ* temperature for sites 792 and 793, hydrothermal waters from Iceland²⁰ and the end-points of sea-water/basalt experiments¹⁷, compared with the solubility of amorphous silica and quartz.

centration of SiO₂ drops from a value close to that of amorphous silica saturation to that of quartz saturation. This is surprising, because natural waters in this temperature range (<80 °C) are generally not in equilibrium with quartz. The data from Reykjanes indicate that temperatures exceeding 200 °C are required for equilibrium (although this may extend down to 130 °C (ref. 19)). But the temperature at which the sudden drop in SiO₂ occurs (indicated by vertical lines in Fig. 4) is different at the two sites. These temperatures coincide with the Miocene/Oligocene boundary, where the rapid increase in Ca occurs. Thus the very low SiO₂ levels are probably caused by precipitation of Ca-containing silicates rather than quartz. This is further substantiated by the fact that at site 793 the concentration of SiO₂ drops below quartz saturation.

The pore waters in the Izu-Bonin fore-arc sedimentary basin are altered more extensively than any recovered previously from deep-sea sediments. This degree of alteration may be attributed to the abundance of volcanoclastic sediments, the very low diffusivity and moderately elevated temperatures in this region. Mass-balance considerations show that between 15 and 25 vol% of the rock matrix has been transformed by low-temperature reactions into zeolites, smectites and gypsum; mineralogical observations are consistent with this interpretation. The reactions have replaced most of the pore-water Na, and almost all of the K and Mg, by Ca. The high Ca concentrations have caused precipitation of gypsum. On the basis of the high Cl concentrations at site 793, water taken up by the secondary minerals can be estimated to correspond to ~26% of the pore space. These observations (changes in the concentration of dissolved species) are consistent with experiments on the reaction between sea water and basalt at comparable temperatures, although water/rock ratios that differ from those in this region can affect the extent of alteration and reverse the trends for pH and SiO₂. □

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- Graf, D. L. et al. *Circ. Illinois State geol. Surv.* **379** (1966).
- Roedder, E. *Prof. Pap. US geol. Surv.* **440-JJ** (1972).
- Livingston, D. A. *Prof. Pap. US geol. Surv.* **440-G** (1963).
- Hardie, L. A. *Contr. Miner. Petrogr.* **82**, 205-213 (1983).
- Frape, S. K. et al. *Geochim. cosmochim. Acta* **48**, 1617-1627 (1984).
- Carpenter, A. B. *Circ. Oklahoma geol. Surv.* **79**, 60-77 (1978).
- Sass, E. & Starinsky, Y. A. *Geochim. cosmochim. Acta* **43**, 885-895 (1979).
- Morton, R. A. & Land, L. S. *Bull. Am. Ass. Petrol. Geol.* **71**, 191-206 (1987).
- Taylor, B. et al. *Proc. ODP init. Rep. 126* (Ocean Drilling Program, College Station, Texas, in the press).
- Gieskes, J. M. in *Chemical Oceanography Vol. 8* (eds Riley, J. P. & Chester, R.) 221-269 (Academic, London, 1983).
- Gieskes, J. M. & Johnson, J. in *Init. Rep. DSDP Vol. 61* (eds Larson, R. L. et al.) 607-611 (US Govt Printing Office, Washington, 1981).
- Krieger, R. A. *Ground Wat.* **1**, 7-12 (1963).
- White, D. E. et al. *Prof. Pap. US geol. Surv.* **440-F** (1963).
- McDuff, R. E. & Gieskes, J. M. *Earth planet. Sci. Lett.* **33**, 1-10 (1976).
- Gieskes, J. M. *Spec. Publs Soc. econ. Palaeont. Miner. Tulsa* **32**, 149-167 (1981).

- Aagaard, P. et al. in *Proc. ODP Sci. Results* **104** (eds Eldholm, O. et al.) (Ocean Drilling Program, College Station, Texas).
- Seyfried, W. E. & Bischoff, J. L. *Geochim. cosmochim. Acta* **43**, 1937-1947 (1979).
- Seyfried, W. E. & Bischoff, J. L. *Earth planet. Sci. Lett.* **34**, 71-77 (1977).
- Arnorsson, S. *Am. J. Sci.* **275**, 763-784 (1975).
- Björnsson, S. et al. *Bull. Am. Ass. Petrol. Geol.* **56**, 2380-2391 (1972).
- Lebedev, L. M. & Nikitina, I. B. *Dokl. Acad. Nauk. SSSR Engl. transl.* **183**, 180-182 (1968).
- Muffler, L. J. P. & White, D. E. *Bull. geol. Soc. Am.* **80**, 157-182 (1969).
- Miller, A. R. et al. *Geochim. cosmochim. Acta* **30**, 341-359 (1966).
- Roedder, E. et al. *Econ. Geol.* **58**, 353-374 (1963).
- Hall, W. E. & Friedman, I. *Econ. Geol.* **58**, 886-911 (1963).
- Edmond, J. M. et al. *Earth planet. Sci. Lett.* **46**, 1-18 (1979).
- Von Damm, K. L. et al. *Geochim. cosmochim. Acta* **49**, 2197-2220 (1985).
- Campbell, A. C. et al. *Nature* **335**, 514-519 (1988).
- Kharaka, Y. K. et al. *Invest. Rep. Wat. Resour. U.S. geol. Surv.* **88-4277** (1988).

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Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein

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Position-effect variegation is the inactivation in some cells of a gene translocated next to heterochromatin, the region of the chromosome that is permanently condensed. The number of copies of the *Drosophila* gene *Suvar(3)7* is a dose-limiting factor in this phenomenon, and seems from its sequence that it encodes a protein with five widely spaced zinc-fingers. This novel arrangement of zinc-fingers could help in packaging the chromatin fibre into heterochromatin, and also reflect a novel method of controlling the expression from DNA domains.

HETEROCHROMATIN is the region of chromatin that does not decondense at interphase. Chromosomal rearrangements that place a gene next to heterochromatin sometimes result in the inactivation of this gene in some cells but not in others. This causes a mosaic or variegated phenotype. Once inactivated, the gene remains so through cell divisions. This phenomenon is called position-effect variegation, and occurs in various species^{1,2}. In mice or humans, for example, the translocation of autosomal regions to an inactivated X chromosome results in the variegation of autosomal genes (for example, see ref. 3). In *Drosophila*, there are dominant mutations that suppress or enhance variegation⁴⁻⁷; the genes in which these mutations occur are termed suppressors or enhancers, respectively. We have cloned one of these modifier genes, *Suvar(3)7* (see refs 8 and 9 and below). We report here that the number of copies of this gene is a dose-limiting factor in variegation and that it encodes a putative zinc-finger protein of a novel type. The five zinc-fingers of the product of *Suvar(3)7* are separated from each other by 40 to 107 amino acids, in contrast with the fingers of other known zinc-finger proteins, which are contiguous. These spaced fingers could make widely separated contacts with the chromatin fibre to form a higher-order complex termed heterochromatin. If the product of this gene participates in the constitution or packaging of heterochromatin, then an increase in its concentration might induce the spreading of the heterochromatic conformation into euchromatic regions. We also speculate that the heritable silencing of defined chromosomal regions by this or analogous mechanisms is an important method of gene regulation during development.

The *Suvar(3)7* gene

The chromosomal inversion *white mottled* (w^{m4}) places the *white* gene, a visible eye-colour marker, next to heterochromatin. The resulting variegation (inactivation) of *white* is easily measured by the decrease in the red-pigment content of the eye. Deletion of one copy of the third-chromosome gene *Suvar(3)7* reduces variegation of the *white* gene in w^{m4} , whereas duplications of

the locus enhance variegation⁸. *Suvar(3)7* also affects variegating rearrangements of other genes⁵, and is one of many loci whose loss has a similar effect¹⁰. Genetic evidence for many such loci^{10,11}, and molecular evidence for *Suvar(3)7* reported here, indicate that they are not redundant genes.

We have mapped *Suvar(3)7* to a previously cloned interval^{8,12}. To delimit the function precisely, we inserted pieces of genomic DNA suspected to contain the gene into the P-element transposon, and microinjected each construct into early embryos. The transposon can integrate in the germ line, and transformants can be recovered in the next generation¹³. We tested the transformants to find whether the inserted DNA fragment enhanced variegation. Figure 1 illustrates a deletion delimiting the gene, and the various clones that we used in P element-mediated transformation experiments. The results are reported in Table 1 and illustrated in Fig 1 and 2. The 10.4-kilobase (kb) *SalI-SalI* fragment clearly enhanced variegation. This result indicates that *Suvar(3)7* lies within the 10.4-kb interval. To further map the gene we repeated genetic rescue experiments with subclones of this 10.4-kb fragment (Fig. 1 and Table 1). Only one subclone—that of the 6.5-kb *EcoRV* fragment—was found to rescue the function of the modifier of variegation.

To establish whether the enhancer effect was due to the position of the insertion, we used a transformant in which a P-element bearing the 6.5-kb *EcoRV* fragment and the *rosy* gene had been inserted into the second chromosome (transformant line T21). We mobilized this insert by introducing transposase activity encoded by the P-element $\Delta 2-3$ on the third chromosome¹⁴, and were able to select seven transformants with new insertions by screening for the *rosy*⁺ marker.

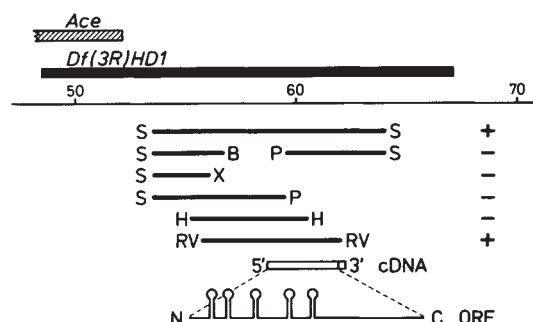


FIG. 1 Genomic DNA fragments tested for their effect on w^{m4} variegation. The coordinates refer to our chromosomal walk⁹. The 5' end of the *Ace* locus³⁴ is represented by a hatched bar. The extent of the deletion *Df(3R)AceHD1* (ref. 35) is marked by a filled bar. The various restriction fragments tested are shown as solid lines. B, *Bam*HI; P, *Pst*I; S, *Sal*I; R, *Eco*RV; X, *Xho*I. The plus and minus signs indicate whether or not the corresponding clones rescue the *Suvar(3)7* mutation. The region covered by the overlapping cDNA clones is represented by an empty bar. The open reading frame (ORF) is enlarged below with a scheme of the putative protein showing the position of the zinc-fingers.

TABLE 1 Rescue of *Suvar(3)7* by genetic transformation

Fragment and transformant line	Chromosomal position	Eye pigment*	<i>R</i> †	Enhancement of variegation
<i>Sall</i> 10.4 kb <i>P</i> [(<i>ry</i> ⁺)10.4 kb]				
T0	48AB	7.4 ± 1.4	0.16	+
T1	26C	10.2 ± 2.9	0.22	+
<i>EcoRV</i> 6.5 kb <i>P</i> [(<i>ry</i> ⁺)6.5 kb]				
T20	3R	4.2 ± 0.7	0.09	+
T21	21AB	10.3 ± 3.0	0.22	+
<i>Sall</i> - <i>XhoI</i> 2.6 kb <i>P</i> [(<i>ry</i> ⁺)2.6 kb]				
T13	33B	55.6 ± 4.2	1.19	—
T36	47B	50.5 ± 8.3	1.08	—
<i>Sall</i> - <i>Bam</i> HI 3.3 kb <i>P</i> [(<i>ry</i> ⁺)3.3 kb]				
T315	3LR	31.9 ± 5.8	0.69	—
<i>Sall</i> - <i>Pst</i> I 5.5 kb <i>P</i> [(<i>ry</i> ⁺)5.5 kb]				
T48	32AF	44.9 ± 6.9	0.96	—
T49	92AF	45.4 ± 3.2	0.97	—
<i>Hind</i> III 5.0 kb <i>P</i> [(<i>ry</i> ⁺)5.0 kb]				
T26	2L	53.7 ± 5.9	1.15	—
T56	56A	39.4 ± 9.6	0.84	—
<i>Pst</i> I- <i>Sall</i> 4.9 kb <i>P</i> [(<i>ry</i> ⁺)4.9 kb]				
T22	26B	46.3 ± 6.6	0.99	—

Genomic fragments used for transformation identified by their size, and the restriction sites used to isolate them are illustrated in Fig. 1. The extent of variegation was quantified in transformed and control flies by measuring the red-pigment content of eyes. Pigment is extracted from heads and extinction determined at 480 nm.

* Percentage of red eye pigments relative to that of control wild-type Canton-S flies. Measurements were made in *w*^{m4}/Y males, heterozygous for the strongly dominant suppressor mutation *Suvar(2)1*⁰¹, and transformant for one copy of the DNA fragment.

† *R* is the ratio of the red eye-pigment content between *w*^{m4}/Y; *Suvar(2)1*⁰¹/+ males with the transformant DNA fragment and *w*^{m4}/Y; *Suvar(2)1*⁰¹/+ control males (control pigment value, 46.6 ± 11.1).

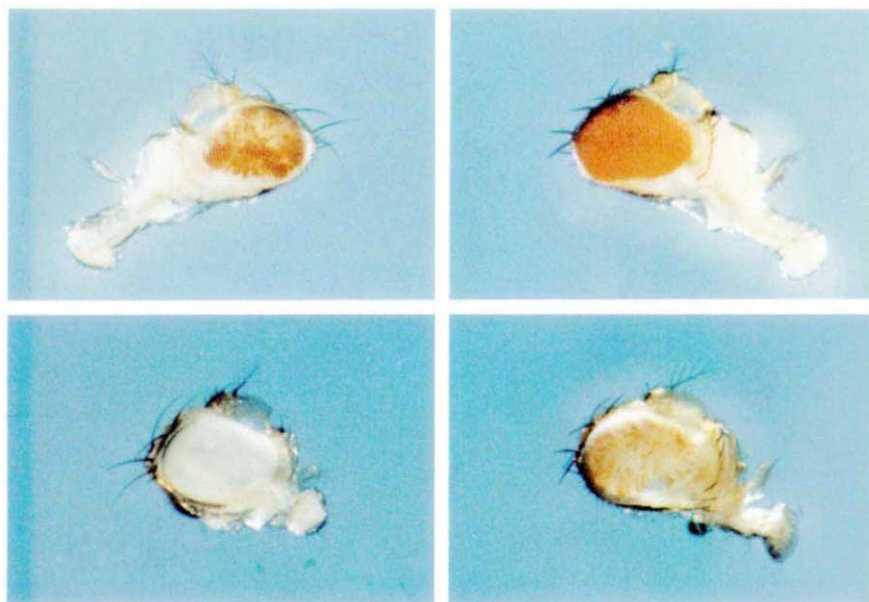


FIG. 2 Enhancement of position-effect variegation by a cloned copy of *Suvar(3)7*. Top left, head of a male fly carrying the *w*^{m4} variegating rearrangement. Inactivation of the *white* gene in some cells early in development is visualized by the white regions in a normally red eye. Bottom left, an extra copy of the *Suvar(3)7* locus introduced by transformation of *w*^{m4} flies with the 6.5-kb *EcoRV* genomic fragment strongly enhanced variegation (inactivation). Top right, head of a male fly carrying both the *w*^{m4} rearrangement and a suppressor mutation *Suvar(2)1*⁰¹. The inactivation (white regions) was suppressed to a large extent. Bottom right, an extra copy of the *Suvar(3)7* locus introduced by transformation with the 6.5-kb *EcoRV* genomic fragment strongly enhanced variegation in the suppressed genotype illustrated in the top right panel.

Two of the new insertions were in the X chromosome, two were in the second chromosome, two were in the third chromosome, and one was in the fourth chromosome. All these new insertions had an enhancer effect on variegation (data not shown). Furthermore, the twelve *rosy*— revertants of T21 that we isolated in this experiment showed no enhancer effect (data not shown).

In conclusion, all these experiments correlate the enhancer effect with the presence of an extra copy of the *Suvar(3)7* locus.

Dose-dependent effect on variegation

We measured the effect of gene dosage in several combinations,

with and without deletion of a copy of the resident gene, together with one, two or three copies of the inserted DNA fragment (Table 2). One copy had a suppressor effect on *white* variegation compared with the effect of two copies (wild type). Three copies had an antipodal enhancer effect, markedly reducing the concentration of red eye pigment (Fig. 2). The product of the gene seems to be a limiting factor controlling variegation. To study the effect of more than three gene copies, it was necessary to suppress variegation to some extent with another modifier locus so that the control (two-copy) concentration of eye pigment was higher than usual. The lower part of Table 2 and Fig. 2 show results obtained after another dominant suppressor of variega-

TABLE 2 Enhancement of variegation with increasing copy number of the *Suvar(3)7* locus

Genotype of w^{m4} males*	Number of copies of <i>Suvar(3)7</i>	Pigment values†	Effect on variegation
$\frac{+}{+}; \frac{+}{+}$	2	2.3 ± 0.9	Control
$\frac{+}{+}; \frac{Df(3R)AceHD1}{+}$	1	32.9 ± 3.7	Suppressor
$\frac{P[(ry^+)10.4 \text{ kb}]}{+}; \frac{+}{+}$	3	0.9 ± 0.5	Enhancer
With <i>Suvar(2)1</i> ⁰¹			
$\frac{Su}{+}; \frac{+}{+}$	2	46.6 ± 11.1	Control
$\frac{Su P[(ry^+)10.4 \text{ kb}]}{+}; \frac{Df(3R)AceHD1}{+}$	2	53.8 ± 12.7	1.15
$\frac{Su P[(ry^+)10.4 \text{ kb}]}{+}; \frac{+}{+}$	3	9.3 ± 3.5	0.20
$\frac{Su P[(ry^+)10.4 \text{ kb}]}{+}; \frac{P[(ry^+)6.5 \text{ kb}]}{+}$	3	8.8 ± 3.2	0.19
$\frac{Su P[(ry^+)10.4 \text{ kb}]}{+}; \frac{Df(3R)AceHD1}{+}$	4	2.3 ± 0.9	0.05
$\frac{Su P[(ry^+)10.4 \text{ kb}]}{+}; \frac{P[(ry^+)6.5 \text{ kb}]}{+}$	5	0.5 ± 0.5	0.01

* Second and third chromosome pairs, respectively. The molecular extent of the deletion *Df(3R)AceHD1* and of the transformed DNA fragments *P[(ry⁺)10.4 kb]* and *P[(ry⁺)6.5 kb]* is given in Fig. 1.

† Percentage of wild-type red eye pigments.

‡ *R* is the ratio of pigment values between the experimental genotype value and control.

TABLE 3 Interaction between the triplo-enhancer effect of *Suvar(3)7* and other suppressors of variegation

Transformants and genotype of w^{m4} males	Suppressor mutations	Map	Eye pigment ratio <i>R</i> *		Resulting phenotype
			T0	T1	
$\frac{P[(ry^+)10.4 \text{ kb}]}{+}; \frac{+}{+}$	<i>Suvar(2)1</i> ⁰¹	2–40.5	0.16	0.13	Variegated
$\frac{P[(ry^+)10.4 \text{ kb}]}{+}; \frac{+}{+}$	<i>Suvar(3)3</i> ⁰³	3–46.6	0.37	0.53	Variegated
$\frac{P[(ry^+)10.4 \text{ kb}]}{+}; \frac{Su}{+}$	<i>Suvar(3)6</i> ⁰¹	3–51.1	0.96	0.97	Suppressed
$\frac{P[(ry^+)10.4 \text{ kb}]}{+}; \frac{+}{+}$	<i>Suvar(3)8</i> ⁰¹	3–53.5	1.05	0.92	Suppressed

* *R* is the ratio of red eye-pigment content between *P[(ry⁺)10.4 kb]/+; Su/+* and *+/+; Su/+* males. If the ratio is significantly <1, the enhancer effect of *P[(ry⁺)10.4 kb]* and the suppressor effect of the mutations interact to form an intermediate phenotype. An epistatic effect of the suppressor mutations is indicated by an *R* value close to 1.

tion, *Suvar(2)1*⁰¹ (ref. 15), had been introduced into the flies. Three copies enhanced variegation, and four copies enhanced it even more. Finally, with a fifth copy, variegation was so strong that pigment could barely be detected. The experiments reported in Table 2 show that there is a gradual enhancement of variegation with an increase in the number of copies of the gene from one to five, and establishes that the suppression of variegation caused by the deletion of a resident copy of the gene can be rescued by the insertion of a cloned copy. The effect of the cloned and transposed gene on variegation cannot be distinguished from that of the original resident gene.

Interaction

It has been estimated from previous screens for modifiers of variegation that the total number of modifier genes exceeds one hundred^{8,10}. Do these genes act through one or many pathways? To address this question, we analysed the interaction of four modifiers of variegation with the *Suvar(3)7* locus by testing whether an additional copy of the *Suvar(3)7* gene (the 10.4kb fragment) could rescue the suppression of variegation due to the deletion of a copy of another suppressor gene (Table 3). The suppression of variegation due to the loss of a copy of *Suvar(2)1*⁰¹ or *Suvar(3)3*⁰³ was rescued by the addition of another copy of *Suvar(3)7*. But this rescue was not seen with

either of the mutants *Suvar(3)6*⁰¹ or *Suvar(3)8*⁰¹. In these cases, suppression of variegation due to the mutation was not reverted by a supernumerary copy of *Suvar(3)7*. This indicates that modifiers of variegation do not participate in only one pathway, but that either different mechanisms or parallel but distinct pathways coexist.

Molecular analysis

We isolated complementary DNA clones from a library made from embryos (M. Goldschmidt-Clermond, unpublished data) using the 10.4-kb *SalI* fragment as a probe. Southern blots with the genomic DNA or with the cDNA clones (ref. 18, and data not shown) reveal only one copy of *Suvar(3)7* in the genome. We sequenced cDNA clones and deduced the putative protein sequence from the coding sequence (Fig. 3). The main feature of the 932-amino-acid deduced protein sequence is five potential zinc-fingers of the Cys₂-His₂ type (see ref. 19 for a review). These are boxed in the Fig. 3. It is intriguing that in the middle of each pair of histidines there is a potential phosphorylation site in the environment favoured by protein kinase C. These constitute only a fraction of the 40-or-so potential phosphorylation sites found throughout the sequence²⁰. We also noted a stretch of glutamic and aspartic acid residues between zinc-fingers three and four. There are additional acidic regions in

ATGTCGCATCTGCAGCGTGGGAACTGAACGTGGAGTTTGTCTATTGGCAACGCCACGAA	60	GCCGAGGCTAGACAGAAAGCGGCTATGCGAGCTGCAACGGAAGTACGACTGGCTGGAT	1500
M S H L Q R A N E R G V C L F A Q R H E		A E A R Q K A A M A E L Q A K Y D W L D	
ACCAACAAAGGTACATGGAGGCACTGGCAACCTAGATTCCGATAAGCGGAGTCCGAAG	120	CCTGATGCCAATGACGAGAATCACTGTCTACTGCCGTGTTTGTATGATCGCGCTGCCGATC	1560
T T K G H M E A L R N L D S D K R S R K		P D A N D E N H C H C R V C D S R L P I	
CGAAAGAGGAGCAAGCAACAGCGTGACCAATAGCGCGGGGATGAGGCGAGCGAGAG	180	AAGGTATTTTACCTGCGGCAACGAGCGCTTCCGTAAGCAGCTCGAAAATAGGAGCGG	1620
R K R S K S N S V T N S G G D E A E R E		K V F Y L R Q H D A S R K H V E N K E R	
AAGGAATCTGAACCAAGCTGGCGGAGGAGTCTCAGGACACCCGTGTGGTTATGATG	240	CAAGGGCGAATGCCGCGCTGCCGCAATGCAACCATGATGATGATGATGATGATGATGATG	1680
K E S E P E A G P E D A Q D T P V V M M		Q R A N A A A A A N A P S V S P T S T V	
AACGGGATGTGGACTCAGGCGACGACCTGCCAAGTGGTGTGCACTGATTCGGACACC	300	GATGCCGAAAGGCGAGGAGTCTGGAATGGACAGGAGAGCGAAACGATATGAGCGTTGCA	1740
N G D V D S G D D P G K W C A L I P D T		D A E R Q E S G M D K E S E N D M S V R	
AATCCACAGCAATGCCGATGTACGCTCTGCAACTGCACATGGCGATTACCGATTCTCTA	360	TCCGATGGCAGCAGCGGAAACCGCTGCCGAAAGCGGTCGCGACGCTCAATGGAGGTTGCT	1800
N P Q Q C R C T L C N C T M A I T S F L		S D G S T A E P L A K R S R R S M E V R	
CGGCACTGTAAACAGGAGCTCATTTGTACATGCTATGACACCCGCTGAAAGGGCTCT	420	CGCATATACGCGCCTTCGCGACTCAATGGCAAGGAGAGGAGAGGAGAGGAGAGGAGAGG	1860
R H C K T R A H C H M L S T P A E K G S		R I I R A L R D S M G K R Q E E R S Q M	
TCAGATATTCGGGCACTTTGGCGGTGTGCGGACATGCATCTTGGTTGATGCTGAT	480	GACATGGCAGGAAATGATATGCTGCTGTTGATATTTGATACGCTTCCGAACTTTCG	1920
S D I R G I W A V F A D M H P W L I A D		D M A R N M I C S S F D I V T R L R T L	
CCGAAAGACCCGCAATGGGTACTGACGCGCTGTGCGAAGGAGTTTCATGTATGAAAC	540	GAACGGGAGTCCGTGCGACATAACGAGTCTATGCCCGGCGCGCCATCGGTGACAGTG	1980
P E D P S I G Y C S V C R K R F M Y G N		E R E S V A H N E S M A Q A P P S V T V	
TCAGAGATCAAGCGCAAGATCAAGAAATCCGAGAGCATACCTTGGCTTGGCCAGC	600	TCACCGATTAAAGCAGCGGAGCCAGACATGTAATGGATCTCTTCTTGACAGCATTTCG	2040
S E I K R K N H E K S E K H T L A L A S		S P I K P P E P R H V M D L F F D S I S	
GCCAAAGCGAGCATGAACTTGGTTCGGCTGACGCGAGAGGCGGATAACATGGATGAA	660	CGACCATGAAGTCCTGCGCGGATTTAGCGCAGAGGCGAAGTGGAAATCATGCAG	2100
A K A G I E V G S A D G R G G D N M D E		P T M K S L P P D L A A E G K S K I M Q	
GAGGAAGCGCGCAAGTGAAGCCAGTATCGCAGACCGATGACAGCGAAGATAAT	720	CTCGTTTGTAGCTTGGAGCTTCGAGCCATGACAGAAATGCGACACTCCAAACCCGAGCT	2160
E E A A A S D Q A Q S S Q T D D S E D N		L V C S L E L R A M Q R N A T T T P T A	
GAGGATGACAAATGGTCCGAGATCCAAAACTCGGAAAGGTTTGTCTATAAGCTCTCT	780	ACAGTTTCTGCTCTCTGAAATGGCTTCTTCAACTACGCTGACCCAGTTAAACTCTCT	2220
D D D N W S E I Q K L G K G F A H K S S		T V S A S S K W P S S T T V T P V K T P	
AGCGAGCAAGAAAGGCGACGCTTGGCGTGGGTACGTTTTCACCGTGGCTTGTCTAC	840	CCTGCTCTATTTACGCTCCCTTGTCTGTCGATGAGCATTGCAATTCCTCGGTTGTC	2280
S E P R K A T V R A G V R F Y P W L C Y		P A P I S A P L A S V D A D L H S S V V	
TCCAAGGACGAAAGACTCAGATTTCGAAATCTGCCGCGTGGCTTTCACACGAAAGG	900	ACAACCGCGACGAATACAATAACGGGAGAAACAATAACGACAGGAAACCGTACCG	2340
S K D R K T Q I C K F C R V R F H N E A		T T P H E Y N N G Q N N N N D K E T V P	
GCAAGGCGCGGCACTGAGTGGCGCGGATGTAAGCTGGTAAGGAGCTTCAAAATG	960	AAAGACCGAGTACCGCGGCTTCTCTGCCAAGTGACCATCAATGGGAGCGCAAGAG	2400
A K A R H E L S A R H V K L V K Q F K M		K E P V T G A S S A Q V T I N G S A K D	
CGCAAGCGAAATCTGACAGGCGCAACACGCAAACTAAACATAATGCGCAGGATGAT	1020	CTGCCAGAGAATATACCGCTATCTTAATCTCCAAACCAAGCAAGTGACCAACCGCTC	2460
R Q A K L H Q G T N T Q T K H N A Q D D		L P E N I R R I L T S N Q T Q V T N R L	
GAAGATCAGAGGAACAGGACGAGGAATATGGAGAAAGAGGAGGACGAGAGAGAT	1080	GAGACGGATTCGCTGCGTGTGTACCTGTTGACAACTAACCAACAGAGTGGAGCAAC	2520
E E S Q E Q D E E Y G E E E D A E E D		E T D S V R C V P V D K L T T Q S R T N	
TCCAAAGCAATTCGACTTGGCCAGCTGCAGGCAAGAAAGACTGCTCGAGCGCAAT	1140	GCGAAGGAGGACTCAGTCAAGGTGGCACTTGGGAAGCACCATCCACCCACAGCGCGAC	2580
S Q S N F D L G T V Q A R K T A R A D N		A N G R L S Q G G T S E A P S T P Q A D	
AAGCTTTTGTCAAGCCATTCGCGCAACATGAAGGCAAGGTTATGGTGTGGAAGGC	1200	CTTAGCAATGGTAACAGTTCGCGCATGATCAGGAGATAGCGGCTAACAAATACCAAGT	2640
K L F V K P I P A T M K G K V M V W K G		L S N G N T L A M I R Q I R A N N N N S	
CGCTTCCGCTGGCTCTCTACAAAAGAGGAGCAGCGTGGAACTATGCTTGTGCAAG	1260	TCCAAGATCAGCTTACTAATACGCTCAGATGCAACAGCGCGAGGAGCAGGCAAGC	2700
R F P W L S Y K K N E Q R G N Y A W C K		S K I T V T N T P Q M Q Q P Q Q A Q A S	
CTGTGGAAGTGTCTCTACCTGCCCTCATCCAAAGTGGGCTCCAAAGCAGCAGGACT	1320	ATAAGTCTCTGACTCCAATATGTGGTGGTCCGAGCAGCAAGGTTGTCAATACCA	2760
L C E V S L Y L P S S K W A S K H Q R T		I T S S T P I M R G G P S S N G C Q I T	
TCCGACACATAGCGCTCGCATTGATCGAAAGCGCAATGGTGGCAACCTTTGAAACA	1380	ACATTCGGCAATGGTGAACCAATACCGAGGCCA	2796
S R H I R L R I D R K R N G G N P L K T		T F R T M V N H N R R P	
TGAAACAAAACCTCAGGTGAATATCCACTGTTGTGGCCACAGCTTCGGCTTGGCCAGC	1440		
S N K N S G E I S T V V A T A S A L A S			

FIG. 3 Nucleotide and deduced protein sequence of the product of the *Suvar(3)7* locus. The sequence was determined from the overlapping cDNA clones. The putative zinc-fingers are boxed, and the landmark cysteines and histidines are outlined. A stretch of glutamic and aspartic acid residue is

underlined. The potential protein kinase C phosphorylation sites detected by computer²⁰ are marked by a triangle; other phosphorylation sites are marked by a dot.

front of finger one, between fingers two and three, between fingers four and five, and just after finger five. We propose that this protein is the product of the modifier of variegation because: (1) the open reading frame encoding it is entirely contained in the right half of the 6.5-kb *EcoRV* fragment that rescues function; (2) the left half and most, but not all, of the rightward sequences of this fragment fail to rescue function, as demonstrated by transformation with a *HindIII* fragment (Fig. 1 and

Table 1) and (3) sequencing of the complete 6.5-kb *EcoRV* genomic fragment (L.M.C. Hall, P. Mason and P.S., unpublished data) reveals no other large open reading frame in either orientation.

Variegation and genetic control

In a comprehensive study of 43 dominant modifiers of variegation, Wustmann and co-workers¹⁰ assigned the mutations to

four groups: dominant suppressor mutants with and without an antipodal enhancer effect of three copies of the gene, and dominant enhancers mutants with and without an antipodal suppressor effect of three copies. The authors proposed that the group of genes comprising *Suvar(3)7* (suppressors with three-copies enhancer effect) plays an important part in heterochromatin constitution or packaging. Locke *et al.*²¹ speculated that heterochromatin is composed of a chain of multimeric complexes that spread by concatenation of new units of the complex; the modifiers of variegation are constituents of the units of the complex. The interesting point of this model is that it invokes an analogy with the chemical law of mass action. The solute concentration of each component is a limiting factor in the concentration of the assembled complex. The dose of each of the modifier gene product will affect the assembly of heterochromatin, and hence the spreading of heterochromatin, and as a consequence the inactivation of neighbouring genes (variegation). This is an attractive explanation for many nonredundant but interacting (additive) functions contributing to the same end-structure.

The five putative zinc-fingers in *Suvar(3)7* are separated by 40 to 107 residues. To our knowledge, such separation has not been reported for other zinc-finger proteins; zinc-fingers of proteins of the transcription-factor class are contiguous¹⁹. We propose that the spacing of fingers in *Suvar(3)7* reflects a new function, such as the attachment to relatively distant sites on DNA for its packaging into heterochromatin. The sites could either be evenly distributed in the periodic organization of the chromatin fibre, or irregularly spaced on the DNA, like the scaffold attachment sites²². Five acidic regions are found next to, or between, the fingers. These stretches are similar to sequences in other proteins, for example, the nonhistone chromosomal proteins nucleoplasmin and members of the high-mobility group (HMG) proteins (see ref. 23 and refs therein), and nucleolin²⁴, a principal constituent of the nucleolus. The polyacidic stretches in nucleoplasmin and HMG proteins are thought to bind histones and transfer them to DNA for nucleosome assembly. Acidic stretches involved in protein-protein interactions have also been proposed for the assembly-dependent activation of multimeric transcription factors (see ref. 19 for a review). We therefore speculate that the product of *Suvar(3)7* interacts with both DNA through the zinc-fingers and proteins, most probably histones, to 'freeze' the euchromatic conformation in a closed heterochromatic structure. Preliminary evidence indicates that the *Suvar(3)7* gene product is indeed located in the cell nucleus (F. Cléard, V. Garzino, A.S. and P.S., unpublished data). It is interesting that a similar acidic stretch occurs in a heterochromatin protein²⁵ that is allelic to a suppressor of variegation²⁶. Another extraordinary feature of the *Suvar(3)7*-encoded

zinc-fingers is that in each of them, the histidine doublet is separated by five amino acids, and the central amino acid is a putative phosphorylation site, providing another possible point for control. Indeed, the protein has about 40 potential phosphorylation sites (Fig. 3). This again is reminiscent of the phosphorylation-dependent assembly of multimeric factors binding to DNA²⁷. If the *Suvar(3)7* gene product is regulated by phosphorylation, suppressors of variegation interacting with *Suvar(3)7* would be expected to also include kinases and phosphatases as epistatic regulators.

Inactivation of relatively large chromosomal domains has been invoked as a mechanism of genetic control in, for example, the regulation of the bithorax complex of homoeotic genes in *Drosophila*²⁸. The successive inactivation of neighbouring domains of DNA along the antero-posterior axis of the embryo could control the regional expression of genes and the realization of the body plan. An argument in favour of a spreading mechanism is that the order of genes is conserved from insects to man (see ref. 29 for a review), although it is claimed that the 'thoracic' and 'abdominal' halves of the bithorax complex can be separated by rearrangements without effects³⁰. In the experiment from which this claim arises however, the two rearrangements overlap and each one includes the connecting region. We also note that the bithorax complex is under the control of a family of dominant *trans*-repressor genes. One of them—*Polycomb*—encodes a protein that binds to the bithorax complex³¹ and acts as a dose-limiting factor, because its effect can be titrated by increasing the copy number of the bithorax complex³². We propose that variegation and the control of homoeotic genes are analogous mechanisms. The mechanisms could even overlap. Preliminary evidence (G.R., J.G. and H. Gyrkovics, unpublished results) indicates that some modifiers of variegation control the expression or maintenance of the determined state of homoeotic genes of the bithorax complex.

In conclusion, we believe that a large set of genes encodes proteins that control gene expression by opening or closing large DNA domains. The closing of a domain would proceed by the cooperative assembly of specific proteins into heterochromatin, or into an analogous structure for homoeotic genes, constituting large invasive multimeric complexes. The transcription of an open domain would then be regulated by the network of the usual enhancer, transcription factors and promoters. Not all of the modifiers would need to be structural proteins. They could be enzymes that modify chromosomal protein to produce a general effect on chromosomal conformation and gene expression³³. Finally, there are classes of modifiers that have an opposite effect, namely the haplo-enhancers of variegation (see ref. 10 and refs therein). These products could be important in establishing or maintaining the active conformation of the chromosome. □

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- Baker, W. K. *Adv. Genet.* **14**, 133-169 (1968).
- Spofford, J. B. in *The Genetics and Biology of Drosophila* Vol. 1c eds. Ashburner, M. & Novitski, E. 955-1018 (Academic, New York, 1976).
- Russell, L. B. *Science* **140**, 976-978 (1963).
- Spofford, J. *Genetics* **57**, 751-768 (1967).
- Henikoff, S. *Genetics* **93**, 105-115 (1979).
- Reuter, G. & Wolff, I. *Molec. gen. Genet.* **182**, 516-519 (1981).
- Sinclair, D. A. R., Mottus, R. C. & Grigliatti, T. A. *Molec. gen. Genet.* **182**, 516-519 (1981).
- Reuter, G. *et al.* *Molec. gen. Genet.* **210**, 429-436 (1987).
- Bender, W., Spierer, P. & Hogness, D. S. *J. molec. Biol.* **186**, 17-33 (1983).
- Wustmann, G., Szidonya, J., Taubert, H. & Reuter, G. *Molec. gen. Genet.* **217**, 520-517 (1989).
- Reuter, G., Dorn, R., Wustmann, G., Friede, B. & Rau, H. *Molec. gen. Genet.* **202**, 481-487 (1986).
- Gausz, J., Hall, L. M. C., Spierer, A. & Spierer, P. *Genetics* **112**, 65-78 (1986).
- Rubin, G. & Spradling, A. C. *Science* **218**, 348-353 (1982).
- Robertson, H. M. *et al.* *Genetics* **118**, 461-470 (1988).
- Reuter, G., Dorn, R. & Hoffmann, H. J. *Molec. gen. Genet.* **148**, 480-485 (1982).
- Candido, E. P. M., Reeves, R. & Davie, J. R. *Cell* **14**, 105-113 (1978).
- Sealy, L. & Chalkley, R. *Cell* **14**, 115-121 (1978).
- Hall, L. M. C., Mason, P. J. & Spierer, P. *J. molec. Biol.* **169**, 83-96 (1983).
- Struhl, K. *Trends biochem. Sci.* **14**, 137-140 (1989).
- Bairoch, A. *PC/Gen Software* (University of Geneva, TM IntelliGenetics Inc. and Genofit SA, 1989).
- Locke, J., Kotarsky, M. A. & Tartoff, K. D. *Genetics* **120**, 181-198 (1988).

- Gasser, S. M. & Laemmli, U. K. *Trends Genet.* **13**, 16-22 (1987).
- Dingwall, C. *et al.* *EMBO J.* **6**, 69-74 (1987).
- Bourbon, H., Lapeyre, B. & Amalric, F. *J. molec. Biol.* **200**, 627-638 (1988).
- James, T. C. & Elgin, S. C. R. *Molec. cell. Biol.* **6**, 3862-3872 (1986).
- Eisenberg, J. C. *BioEssays* **11**, 14-17 (1989).
- Yamamoto, K. K., Gonzalez, G. A., Biggs III, W. H. & Montminy, M. R. *Nature* **334**, 494-498 (1988).
- Peifer, M., Karch, F. & Bender, W. *Genes Dev.* **1**, 891-198 (1987).
- Lewis, J. *Nature* **341**, 382-383 (1989).
- Struhl, G. *Nature* **308**, 454-457 (1984).
- Zink, B. & Paro, R. *Nature* **337**, 468-471 (1989).
- Duncan, I. & Lewis, E. B. in *Developmental Order: Its Origin and Regulation* (ed. Subtelny, S.) 533-554 (Liss, New York, 1982).
- Dorn, R., Heymann, S., Lindigheit, R. & Reuter, G. *Chromosoma* **93**, 398-403 (1986).
- Fournier, D., Karch, F., Bride, J.-M., Hall, L. M. C., Bergé, J.-B. & Spierer, P. *J. molec. Biol.* **210**, 15-22 (1989).
- Nagoshi, R. N. & Gelbart, W. M. *Genetics* **117**, 487-502 (1987).

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IC342 is a nearby late-type (Scd) spiral galaxy at a distance of 3.9 Mpc (ref. 1). The galaxy lies nearly face-on—it has an inclination of 25° (ref. 2)—and thus presents an ideal opportunity for the study of galactic kinematics and composition. The central region shows strong mid- to far-infrared emission from warm dust³, and a radio continuum (Fig. 2*b*, *c*) of predominantly thermal origin⁴, indicating the presence of young massive stars and of recent or present star formation in the central region (that is, nuclear starburst activity)³. Single-dish observations with resolutions larger than $45''$ have revealed that CO($J = 1 \rightarrow 0$) line emission is strongest at the nucleus^{5,6}. Interferometric observation of the CO line has revealed the presence of a bar-like structure of molecular gas⁷.

We performed an aperture synthesis mapping of CO ($J = 1 \rightarrow 0$) line emission (rest frequency = 115.271 GHz) from the central region of IC342 with the Nobeyama Millimeter Array (NMA)⁸ during the period December 1988 to April 1989. The array comprises five 10-metre antennas. The primary beam size is 65'' in HPBW (half-power beam width). The maps shown here are not corrected for primary beam attenuation. The antennas are equipped with cryogenically cooled mixer receivers with superconductor-insulator-superconductor junctions. The system noise temperatures in the single side band were ~ 600 K toward the zenith. We used a fast-Fourier-transform spectro-correlator, FX⁹, with 1,024 channels which allowed us to cover a frequency range of 320 MHz (corresponding to 831 km s⁻¹ at 115 GHz). Four array configurations were used and the source was observed twice for each array configuration. We have achieved an angular resolution of $2.4'' \times 2.3''$ (HPBW) which corresponds to a linear

FIG. 1. Channel CO intensity maps integrated over a velocity width of 4.87 km s^{-1} . The maps were processed using CLEAN¹⁰ in the Astronomical Image Processing System installed in the Nobeyama Radio Observatory. The central velocity of each map is denoted in the upper-right corner in each frame. The r.m.s. noise level in each channel map is $\sigma = 1.7 \text{ K}$. The synthesized beam is shown in the upper-right corner of the map at -21.8 km s^{-1} . No corrections are made for primary-beam attenuation. The lowest contour level and subsequent contour intervals are 3.5 K in brightness temperature. The maximum brightness temperature in the channel maps is 20 K . The cross denotes the peak in $2.2\text{-}\mu\text{m}$ emission found in ref. 3; this corresponds to the nucleus (RA (1950) = $3^{\text{h}}41^{\text{m}}57.2^{\text{s}}$, dec. (1950) = $67^{\circ}56'27''$). The ellipse denotes the peaks of radio continuum emission in Fig. 2c. The angle of field of view is RA (1950) = $3^{\text{h}}41^{\text{m}}57.0^{\text{s}}$, dec. (1950) = $67^{\circ}56'30''$.

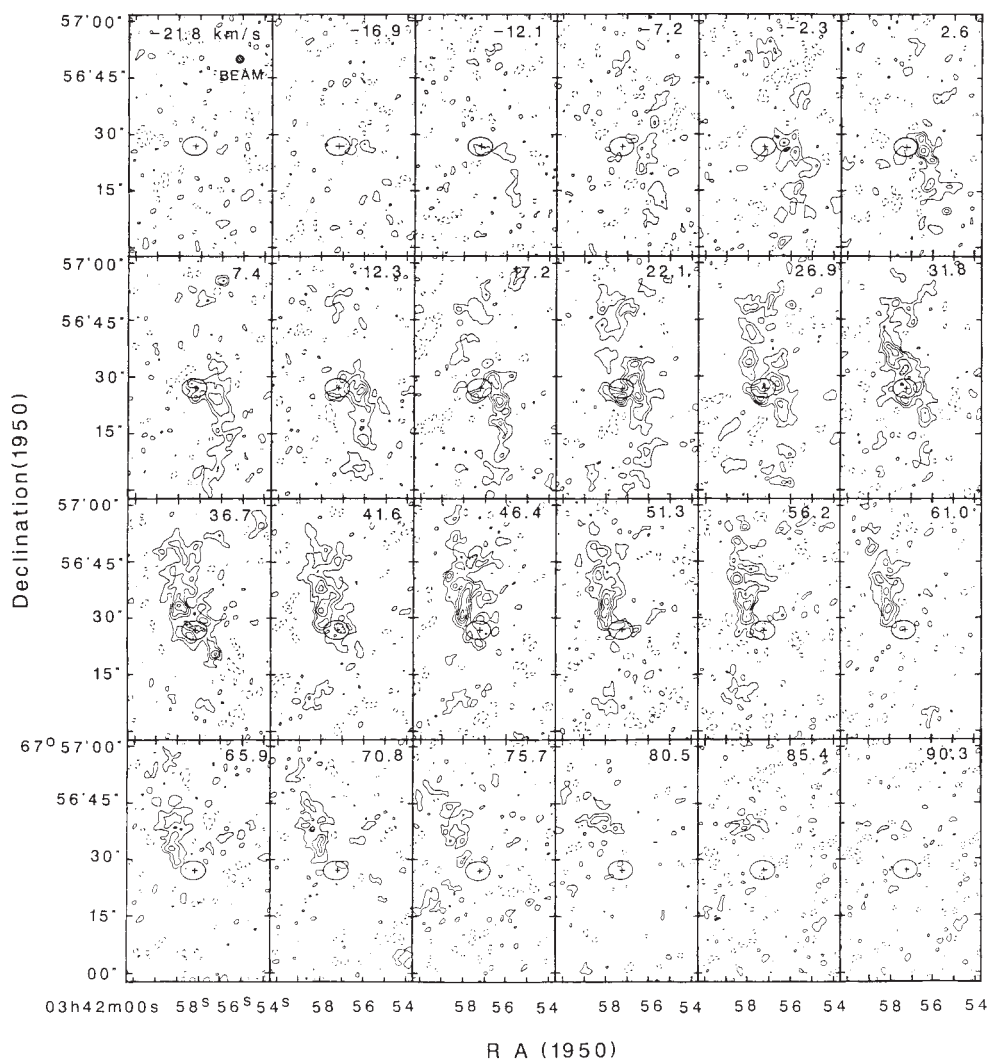
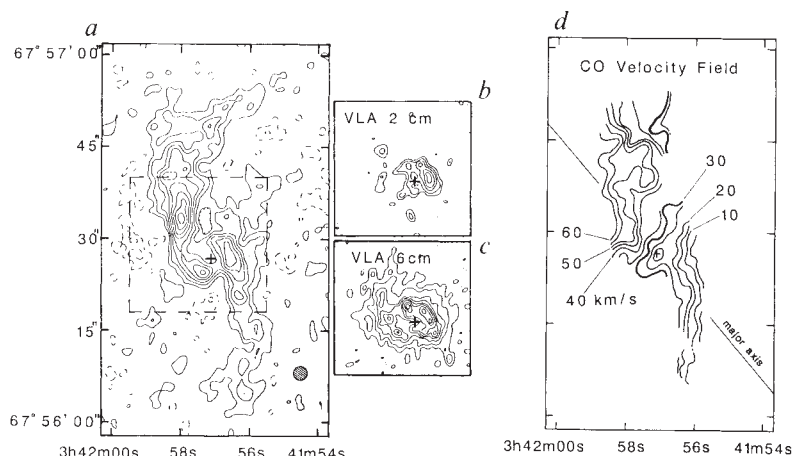


FIG. 2 *a*, Map of integrated intensities obtained by summing all channel maps in Fig. 1. The lowest contour level and subsequent contour intervals are 89 K km s^{-1} . The dashed box corresponds to the area in *b* and *c*. The synthesized beam is shown in the lower right-hand corner. In all maps, the crosses are the same as in Fig. 1. *b*, *c*, VLA (Very Large Array) maps of 2- and 6-cm radio continuum emission⁴. The relative positional error between the CO map (*a*) and radio continuum maps (*b*, *c*) is smaller than $1''$. The total flux densities are 38 and 82 mJy, respectively. *d*, Contour map of the CO weighted mean velocities, $\sum v T_b(v) / \sum T_b(v)$, with spatial smoothing. The summations are over all channel maps in Fig. 1, and brightness temperatures less than 3σ in every channel map have been clipped. The figures indicate the mean velocities with respect to the local standard of rest, V_{LSR} , in km s^{-1} . The systemic velocity, $V_{\text{LSR}}^{\text{sys}} = 30 \text{ km s}^{-1}$ (ref. 2), is shown by thick solid curves. The major axis of the galaxy is determined from observations of the 21-cm line from neutral hydrogen (H I) over the whole galaxy². The inclination of the galaxy to the face-on configuration is 25° (ref. 2). The H I velocity field shows that the northwestern side is the near side if the spiral arms of the galaxy are trailing arms.



scale of 45 pc at a distance of 3.9 Mpc. The intensity scale was referred to 3C84, the flux density of which (14 Jy) was calibrated by observing Mars and Uranus. The intensity scale includes an uncertainty of about $\pm 15\%$. Spectral-line channel maps are shown in Fig. 1, and a map of integrated CO intensity (Fig. 2*a*) was made by combining these channel maps.

The total CO flux in the field of view is $(1.7 \pm 0.3) \times 10^3 \text{ Jy km s}^{-1}$ after correcting primary beam attenuation. This value is about 86% of that measured in ref. 5 with the NRAO 11-m antenna corrected for forward coupling efficiency. Using the normal conversion relation between CO brightness temperature and surface density of molecular hydrogen, for the case of an optically thick CO ($J = 1 \rightarrow 0$) line⁶, the total H_2 mass in the field of view is estimated to be $2.6 \times 10^8 M_\odot$. This conversion relation has been estimated for cold molecular clouds in the disk of our galaxy, and includes several uncertainties; for Galactic molecular clouds, it has an uncertainty probably larger than a factor of two⁶. Furthermore, a higher gas temperature, as suggested from our observations (see below), and the higher metal abundance expected for starburst regions would lower the conversion factor, and the CO emission would be partially optically thin. Thus the uncertainty of our estimate of H_2 mass may be much larger than a factor of two.

The most prominent features in the map of integrated CO intensity (Fig. 2*a*) are two narrow ridges running north-south. They are offset and separated by $\sim 9''$. The northern ridge is more prominent than the southern one. The ridges are $\leq 4''$ (80 pc) wide and $\sim 25''$ (500 pc) long. They may in fact be longer than is evident in the maps because CO emission outside the $65''$ field of view is attenuated by the antenna response. Previous observations have revealed the presence of bars of molecular gas within the nuclei of galaxies NGC6946¹¹, IC342⁷ and Maffei 2¹², and also offsets of the CO ridges in IC342⁷ and Maffei 2¹². But the narrow ridges and their offsets could not be seen so clearly. Here we have resolved such a gas bar into a pair of narrow CO ridges. Comparison with an image at B band shows that the CO ridges are associated with dark lanes¹³.

The CO ridges continue into the nuclear region where they bend to form a ring-like structure with a diameter of $\sim 6''$ (110 pc at a distance of 3.9 Mpc) surrounding the nucleus. There is a 'hole' in the CO emission at the nuclear centre. A comparison of Fig. 2*a* and *c* shows that the ring-like structure of CO emission coincides with a similar structure in the 6-cm radio continuum emission⁴ (a possible site of starburst activity)³ within an accuracy of ~ 1 arcsec.

Figure 1 shows that the CO ridges are composed of a number of clumps several tens of parsecs in size. These may be

giant molecular clouds (GMCs) or associations of GMCs. The channel maps show that the peak brightness temperatures of the clumps are typically 15 K; in the northern ridge there is a maximum of 20 K. This should be compared with typical GMC temperatures of ~ 10 K in our own galaxy¹⁴.

The CO velocity field is shown in Fig. 2*d*. Within the CO ridges, the isovelocity contours are parallel to the ridges and inclined away from the minor galactic axis (which is determined from the kinematics of atomic hydrogen within the whole galaxy). If the gas were rotating in a circle in the disk plane, the isovelocity contours would be parallel to the minor axis, so our results indicate that the motion of the gas is non-circular in the ridges. This is consistent with the observations in ref. 7. On the other hand, within the ring structure, isovelocity contours are parallel to the minor galactic axis of the galaxy, so here motion is circular. The CO ridges show a large velocity dispersion, particularly the northern one. The full widths at half maximum of the velocity distribution averaged over a 45-pc area (a synthesized beam) are 30–50 km s^{-1} in the northern ridge, after subtracting the effect of the velocity gradient, compared with $\sim 20 \text{ km s}^{-1}$ in the CO ring-like structure or in the place with weak CO intensity which is $\sim 10''$ north of the nucleus.

As the thermal radio continuum emission is confined to the central ring-like structure⁴, heating of the molecular gas in the CO ridges cannot be due to OB stars. Our results are consistent with the idea that the CO ridges are associated with shock waves formed in response to a bar-like gravitational potential field^{7,15–17} and that the gas at the CO ridge is heated through shock dissipation of the kinetic energy (or dissipative cloud-cloud collisions) that fuels nuclear starburst^{15–17} in the ring. The optical morphology¹⁸ and a 2- μm emission³ of IC342 suggest that the stellar component may be oval; Zaritsky and Lo¹⁹ have suggested that a non-axisymmetric nuclear bulge could be a common feature in spiral galaxies even when there is no obvious photographic evidence for bars. Further study is required, however, to understand better the formation mechanism of the CO ridges. $\square$

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1. Tully, R. B. *Nearby Galaxy Catalog* (Cambridge University Press, 1988).
2. Rogstad, D. H., Shostak, G. S. & Rots, A. H. *Astr. Astrophys.* **22**, 111–119 (1973).
3. Becklin, E. E. *et al. Astrophys. J.* **236**, 441–447 (1980).
4. Turner, J. L. & Ho, P. T. P. *Astrophys. J.* **268**, L79–L83 (1983).
5. Rickard, L. J. & Palmer, P. *Astr. Astrophys.* **102**, L13–L16 (1981).
6. Young, J. S. & Scoville, N. *Astrophys. J.* **258**, 467–489 (1982).
7. Lo, K. Y. *et al. Astrophys. J.* **282**, L59–L63 (1984).
8. Ishiguro, M. *et al. Proc. Int. Symp. Millimeter and Submillimeter Wave Radio Astronomy* (ed. Gomez-Gonzales, J.) 75–84 (URSI, Granada, 1984).
9. Chikada, Y. *et al. Proc. IEEE* **75**, 1203–1210 (1987).

10. Högbom, J. A. *Astr. Astrophys. Suppl.* **15**, 417–426 (1974).
11. Ball, R. et al. *Astrophys. J.* **298**, L21–L25 (1985).
12. Ishiguro, M. et al. *Astrophys. J.* **344**, 763–769 (1989).
13. Ables, H. D. *Publ. U.S. Naval Obs. Ser.* **20**, Part 4 (1971).
14. Scoville, N. Z. & Sanders, D. B. *Interstellar Processes* (ed. Hollenbach, D. J. & Thronson, H. A.) 21–50 (Reidel, Dordrecht, 1987).
15. Spørensen, S.-A., Matsuda, T., & Fujimoto, M. *Astrophys. Space Sci.* **43**, 491–503 (1976).
16. Roberts, W. W., Huntley, J. M. & van Albada, G. D. *Astrophys. J.* **233**, 67–84 (1979).
17. Combes, F. & Gerin, M. *Astr. Astrophys.* **150**, 327–338 (1985).
18. de Vaucouleurs, G., de Vaucouleurs, A. & Corwin, H. G. *Second Reference Catalogue of Bright Galaxies* (University of Texas Press, Austin, 1976).
19. Zaritsky, D. & Lo, K. Y. *Astrophys. J.* **303**, 66–75 (1986).

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Intermittent vortex structures in homogeneous isotropic turbulence

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ALTHOUGH well developed turbulence exhibits complex, chaotic spatial and temporal behaviour, there is much experimental evidence to suggest that a remarkable degree of coherence is also present¹. It is known² that correlations in small-scale turbulent motions show significant deviations from the gaussian statistics usually expected for large, randomly interacting systems. This phenomenon, known as intermittency, has long resisted analytical description because of the lack of a simple, universal characterization of turbulence structures³. Here we report numerical simulations which show that there are remarkably simple spatial structures associated with intermittent regions of vorticity. In particular, in contrast to the classical description of turbulence as an array of 'pancake'- or 'lasagne'-like eddies⁴, we find that high-amplitude vortex structures are tube-like and that they generate local velocity fields that spiral around them.

Structures in turbulence are strongly coherent regions of flows embedded in a highly disordered background. Gaussian statistics are associated with random interactions in large systems according to the law of large numbers, so non-gaussian intermittent behaviour is a signature of coherence in turbulence. More specifically, the statistics of small-scale fluctuations in turbulence differ from gaussian by having a higher probability for high-amplitude events (of vorticity, for example), which contribute primarily to the values of high-order statistical moments. Current theories^{5,6} that approximate low-order correlations by invoking closure relations are able to give a faithful description of these correlations without specifically taking structures into account. It follows that coherent small-scale intermittent structures in turbulence may occupy only a small portion of flow systems and may be built only upon high-amplitude events. Our aim here is to investigate the spatial patterns associated with these intermittent structures, which may contribute to understanding of the dynamical basis of turbulence.

One of the key quantities that characterizes small-scale turbulence is the vorticity $\omega = \nabla \times \mathbf{v}$, which is governed by the dynamical equation

$$\frac{D\omega_i}{Dt} = s_{ij}\omega_j + \nu \nabla^2 \omega_i$$

where $D/Dt = \partial/\partial t + \mathbf{v} \cdot \nabla$ is the convection derivative and $s_{ij} = \frac{1}{2}(\partial u_i/\partial x_j + \partial u_j/\partial x_i)$ is the rate-of-strain tensor. This equation shows that vorticity undergoes two physically significant processes, namely stretching due to s_{ij} and tearing (reconnection) at small scales induced by viscosity.

It was argued by Betchov⁴ that the symmetric matrix s_{ij} is likely to have two positive eigenvalues (the sum of all three being zero as a result of the incompressibility constraint). This speculation was confirmed in numerical simulations of isotropic turbulence^{7,8}. On this basis Betchov⁴ and Kerr⁸ speculated that typical small-scale turbulence structures should be sheet-like, because an initially nearly spherical fluid element will evolve, under the action of a strain with two stretching and one compression directions, to a 'lasagne'-shaped object.

This argument for sheet-like structures requires the assumption of a uniform and constant strain field over the spatial extent of the structures. But strong coherent vortex structures usually exist on scales over which the variation of the strain field is not negligible. More importantly, the intensity of vorticity in some particular element of fluid changes in time as a result of stretching and compression, and the vorticity of this element will in turn modify the strain field surrounding it, especially when the vorticity intensity becomes high. Numerical evidence of the importance of this last effect has been reported recently by She et al.⁹, who showed that alignment of the vorticity vector with the middle eigenvector of the strain-rate matrix⁷ may in fact be due to increasing domination of the strain field by the high-vorticity elements themselves. Thus, high-amplitude (intermittent) vortex structures may show new features that are caused by self-induced stretching, as we effectively observe below.

We have conducted direct numerical simulations of isotropic turbulence based on solutions of the Navier–Stokes equations, by using a pseudo-spectral code with resolution 96^3 in a cube with 2π -periodic boundary conditions, so that wavenumbers $|k| < 48$ are resolved. Using a force that maintains a constant energy level in the first two wavenumber shells ($|k| \leq 2$), a stationary turbulence field with Taylor microscale Reynolds number $R_\lambda \approx 90$ is obtained after a transient period of several large-eddy turnover times ($t \geq 10$). R_λ is a measure of the strength of the nonlinear interaction in turbulence; this regime corresponds to well developed turbulence. Three-dimensional visualizations have been performed on vortex lines obtained by integrating along the vorticity vector from a given set of points. This line-perspective technique is distinguished from previous visualization techniques^{10,11} by its ability to show both the intensity (represented by colour) and the orientation properties of a vector field such as the vorticity. Both of these characteristics are dynamically significant. Vortex lines themselves have additional topological significance¹², because in the inviscid limit their topological structure is invariant.

In Fig. 1a we show a view of vortex lines containing points of high vorticity intensity ($|\omega| \geq 4|\bar{\omega}|$) for the full computational domain. The most striking feature is the high degree of coherence: large-scale, essentially straight tube-like structures dominate the intermittent regions. It is also notable that the structures are distributed sparsely in space, consistent with the earliest observations of spatial intermittency¹³. Tube-like objects associated with high vorticity amplitudes were first noticed by Siggia^{10,11} using a scalar visualization of vorticity intensity; however, the presence of vortex tubes can be demonstrated only by displaying vortex lines. In Fig. 1b we show a close-up view of one of the more prominent structures in Fig. 1a. Here it may be clearly seen that the central, highest-vorticity-amplitude portion of the structure consists of a tube in which the vortex lines are directed primarily along the tube axis. The width of the tube structures is typically of the order of the Kolmogorov dissipation-length scale, the scale at which molecular dissipation becomes important. The intensity of the vorticity gradually decreases along the tube axis; as the intensity falls, the vorticity vectors develop more complex, chaotic orientations. An additional interesting feature is that vortex lines are fairly straight in the core region of the tube but wrap around the tube in the outer portion, forming a spiral pattern.

As discussed above, the form of small-scale intermittent structures may be determined largely by the strain field induced by

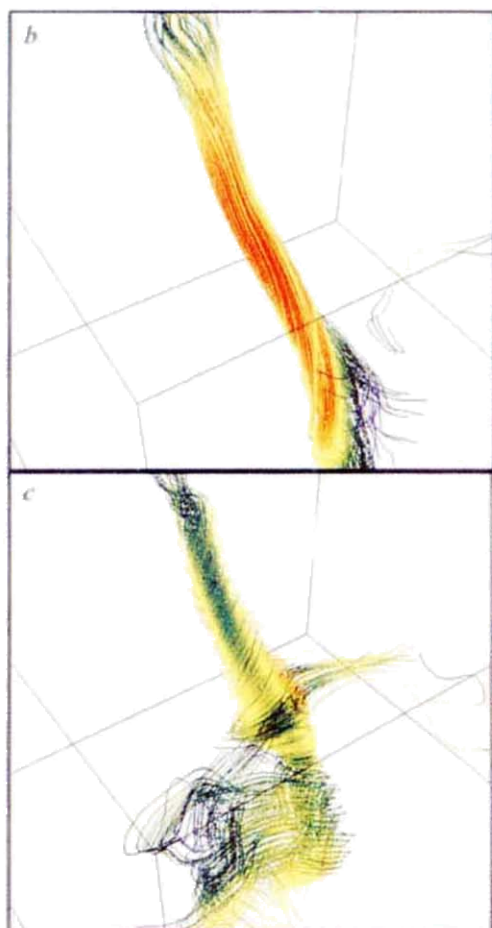
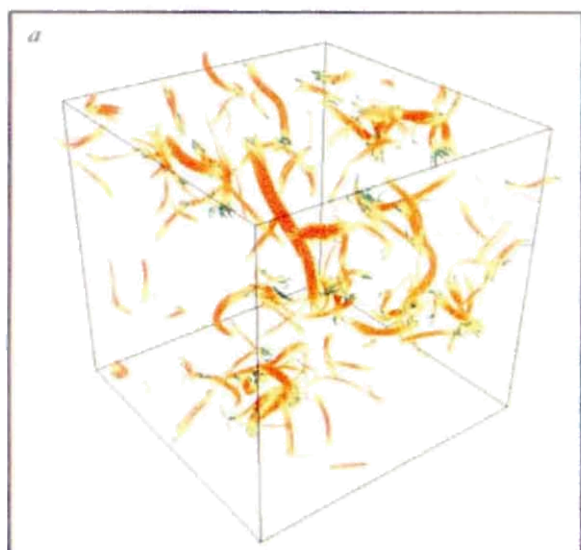


FIG. 1 Three-dimensional perspective view of vortex structures in an instantaneous field of stationary isotropic turbulence obtained by direct numerical simulation. *a*, Global view of vortex lines that pass through points of high-vorticity intensity ($|\omega| > 4|\bar{\omega}|$). *b*, Close-up view of a large vortex tube in *a*. *c*, Close-up view of the velocity streamlines passing through the same set of grid points as the vortex tube in *b*. Colours represent local intensity of the vorticity (*a* and *b*) and velocity (*c*); red-high, green-medium, blue-low.

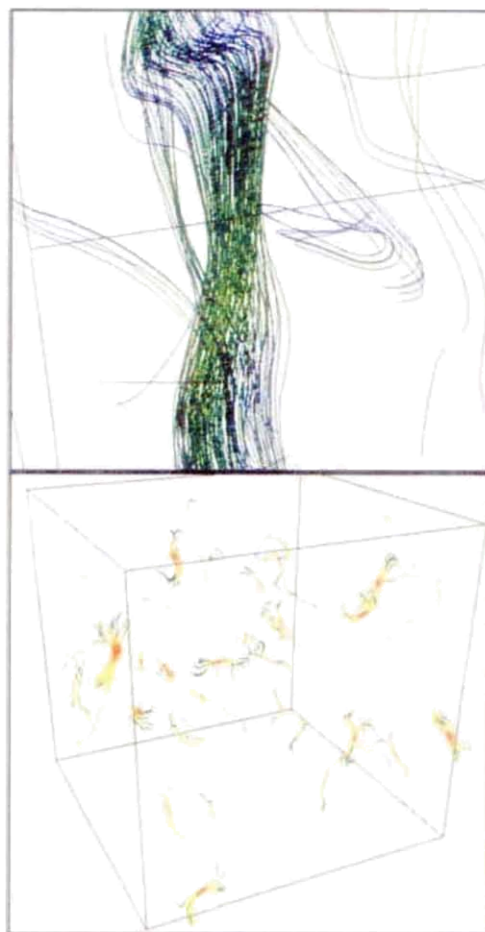


FIG. 2 (Upper frame) Close-up view of sheet-like topology of vortex lines passing through points of moderate ($2.5|\bar{\omega}| < |\omega| < 3|\bar{\omega}|$) vorticity.

FIG. 3 (Lower frame) View analogous to that in Fig. 1*a* performed on a Gaussian random velocity field with an energy spectrum identical to that of the turbulent case.

the local structure itself. In Fig. 1*c* we visualize the velocity streamlines in the region of the vortex tube of Fig. 1*b*. The velocity here comprises essentially a two-dimensional swirling motion around the vortex tube, with a significant component directed along the tube axis. This structure leads to interesting dynamical consequences, namely that the nonlinear interaction $\mathbf{u} \times \boldsymbol{\omega}$ will be directed predominantly perpendicular to the tube axis. This will yield strong divergence which is then cancelled by the pressure, so that the effective nonlinear interaction here is small¹⁴⁻¹⁷. This might also explain the long lifetime observed for these structures.

We have also considered the structure of the vorticity field in regions of moderate vorticity intensity ($2.5|\bar{\omega}| \leq |\omega| \leq 3|\bar{\omega}|$). Here we observe a strong tendency towards formation of sheet-like or lasagne-like structures; a typical example is shown in Fig. 2. The large-scale coherence of these moderate-amplitude structures is still striking, but the behaviour is more complex than for the tubes, with sheets bending and twisting in space. Preliminary studies of the velocity structure in these regions demonstrate a tendency for the velocity to be parallel to the sheet and perpendicular to the vorticity. If so, this should provide a consistent explanation for depletion of nonlinearity in turbulent flows. A detailed investigation of this and of the process of tube development is currently underway.

Finally, it should be noted that at low amplitudes the behaviour of vortex lines is highly random, with no apparent structure. This is consistent with the fact, noted above, that statistical closure theories are able to describe low-order statistical properties of turbulent fields. To emphasize the significance of the geometrical features associated with high amplitudes in turbulence, we have generated a gaussian random velocity field with an energy spectrum identical to that of the turbulent field shown in Fig. 1. In Fig. 3 we show the vortex-line pattern associated with this field. It is clear that there are no large-scale vortex structures similar to those observed in turbulence.

Thus, in isotropic incompressible turbulence at moderately high Reynolds number, highly intermittent vortex structures are typically tube-like. Coherence of the vorticity field continues to be strong at somewhat lower amplitude thresholds as well, but then tends to be associated with sheet-like structure. A preliminary investigation has demonstrated that these structures are fairly long-lived and therefore are likely to play a significant dynamical role. A deeper understanding of the physical processes leading to the formation of these structures and their dynamical

significance is necessary to further elucidate the structural properties of turbulence at high Reynolds number. □

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1. Hussain, A. K. M. F. *J. Fluid Mech.* **173**, 303–356 (1986).
2. She, Z.-S., Jackson, E. & Orszag, S. A. *J. Sci. Comput.* **3**, 407–434 (1988).
3. Frisch, U. & Orszag, S. A. *Physics Today* 24–32 (January 1990).
4. Betchov, R. *J. Fluid Mech.* **1**, 497–504 (1956).
5. Kraichnan, R. H. *Phys. Fluids* **9**, 1728–1752 (1966).
6. Yakhot, V. & Orszag, S. A. *J. Sci. Comput.* **1**, 3–51 (1986).
7. Ashurst, W. T., Kerstein, A. R., Kerr, R. M. & Gibson, C. H. *Phys. Fluids* **30**, 2343–2353 (1987).
8. Kerr, R. M. *Phys. Rev. Lett.* **59**, 783–786 (1987).
9. She, Z.-S., Jackson, E. & Orszag, S. A. *J. Fluid Mech.* (submitted).
10. Siggia, E. D. *J. Fluid Mech.* **107**, 375–406 (1981).
11. Hosokawa, I. & Yamamoto, K. *J. phys. Soc. Japan* **58**, 20–23 (1989).
12. Moffatt, H. K. *J. Fluid Mech.* **159**, 359–378 (1985).
13. Batchelor, G. K. & Townsend, A. A. *Proc. R. Soc. A* **199**, 238–255 (1949).
14. Pelz, R., Yakhot, V., Orszag, S. A., Shtilman, L. & Levich, E. *Phys. Rev. Lett.* **54**, 2505–2508 (1985).
15. Yakhot, V. & Orszag, S. A. *Nucl. Phys. B (Proc. Suppl.)* **2**, 417–427 (1987).
16. Kraichnan, R. H. & Panda, R. *Phys. Fluids* **31**, 2395–2399 (1988).
17. Shtilman, L. & Politke, W. *Phys. Fluids* **A1**, 778–779 (1989).

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Anchoring structure of smectic liquid-crystal layers on MoS₂ observed by scanning tunnelling microscopy

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THE molecular alignment of liquid crystals on solid substrates is important both to fundamental issues in physics and biology and in practical applications such as optoelectronic devices. The structure of adsorbed phases is determined to a large extent by the balance of molecule–molecule and substrate–molecule forces at the solid–adsorbate interface. Scanning tunnelling microscopy (STM) now allows the possibility of direct observation of the interfacial structure with molecular resolution, and has been used to study positional and orientational order in monolayers of organic molecules on graphite^{1–4}. Here we report STM imaging of smectic liquid crystals (4'-n-octyl-4-cyanobiphenyl (8CB)) condensed on a molybdenum disulphide single crystal. The anchoring structure is different from that on graphite⁴, and is unusual in that the molecules are aligned so that intermolecular interactions are energetically unfavourable. The influence of interactions with the substrate is clearly important in this case, and this is further supported by an apparent degree of epitaxy in the adsorbed layer.

STM images have been obtained for a variety of surface-adsorbed organic species, including biological molecules^{5,6}, organic conductors^{7,8} and liquid crystals^{1–4}. The highest-resolution images of molecular packing obtained so far have been for smectic liquid crystals; for example, Smith *et al.*⁴ have studied n-alkyl cyanobiphenyl (nCB) molecules deposited on graphite, and reported two-dimensional molecular packing similar to the nCB smectic bilayers that are found in the bulk liquid-crystal phase. A larger spacing between the 'bilayers' (essentially 'double rows' in the adsorbed phase) and, correspondingly, only a small degree of interdigitation of the biphenyl head groups

and alkyl tails was observed relative to the bulk structure (as deduced from X-ray and neutron-scattering measurements⁹). These high-resolution STM images have so far been restricted to a graphite substrate.

In Fig. 1a we show our own STM image of 8CB in air at room temperature, adsorbed on highly oriented pyrolytic graphite; our results confirm those of ref. 4. It has been reported that a variety of surface phases can exist for 8CB on graphite², but we find that reproducibility depends on preparing good single-domain samples. In particular, it is known that external influences such as high voltage, pressure and temperature can drastically change the nature of the ordered structure, creating defects which induce multi-domain rather than single-domain characteristics for most liquid crystals. Therefore, careful sample preparation is essential to avoid deformation of the intrinsic anchoring structure. Our samples were prepared by heating a drop of 8CB into the isotropic phase on an air-cleaved surface of graphite and then cooling down near the phase-transition temperature slowly enough to allow spontaneous thermal fluctuations to create a well-aligned structure. A platinum/iridium STM tip was scanned over the 8CB layer at a constant tunnel current of 0.09 nA. Care was taken to avoid high-voltage pulses between the tip and sample. The bias voltage of 1.2 V (tip positive) was held constant; no images could be obtained when the polarity was varied. The two-dimensional ordered structure of 8CB on graphite obtained under these conditions is in good agreement with previous results (ref. 4 and V. B. Elings and K. Kjoller, personal communication). The molecular model for this structure is shown in Fig. 1b.

To investigate the dependence of the anchoring structure of nCB molecules on the properties of the underlying substrate, we replaced the graphite substrate with single-crystal MoS₂, which was chosen because heteroepitaxial growth of organic monolayers has been observed previously on this substrate¹⁰. MoS₂ is a typical layered transition-metal dichalcogenide (MX₂); the cleaved surface exposes hexagonally packed sulphur atoms which have no dangling bonds. 8CB was deposited on the air-cleaved surface in the same manner as for graphite.

The STM image of the anchoring structure of 8CB layers on MoS₂ is shown in Fig. 2a at a voltage of -0.75 V and a tunnel current of 0.35 nA. In contrast to the 'bilayer' structure on graphite, 8CB on MoS₂ exhibits a periodic 'monolayer' ('single-row') structure in which cyanobiphenyl head groups and long alkyl tails alternate in each row. In numerous such studies on MoS₂ we find no evidence of packing structures other than this one. This periodic structure comprises a large homogeneous single domain over an area of 500 × 500 Å, approaching the

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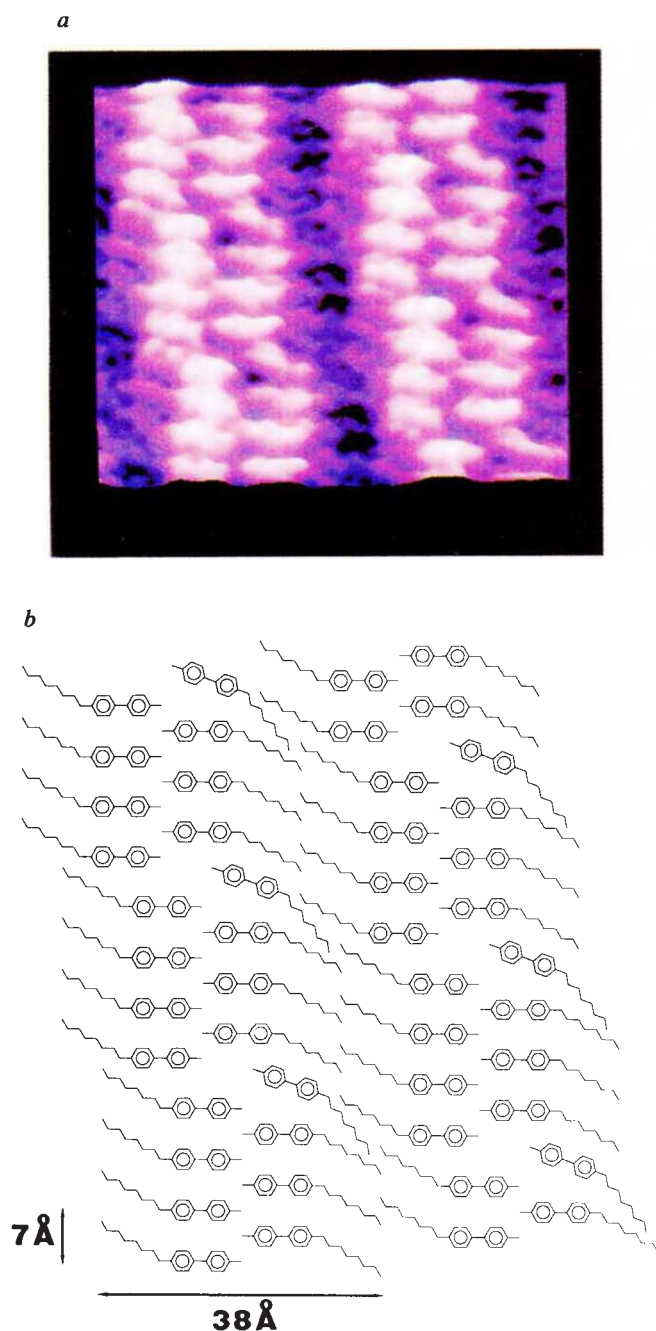


FIG. 1 *a*, STM image of 8CB on graphite. Area of image is $80 \times 80 \text{ \AA}$. The carbon atoms of the alkyl chains are dark and the aromatic groups are bright. Along each row there is a repeating unit four molecular pairs in length, with every fourth alkyl tail dipping towards the surface. *b*, Model showing the 'bilayer' packing of 8CB molecules on graphite. Every fourth molecule in one of the two rows has a different orientation to the others.

limiting area that the STM can scan.

Figure 2*b* shows the molecular packing structure of 8CB on MoS_2 . Each row has a width of $\sim 21 \text{ \AA}$, which corresponds to the length of an 8CB molecule, whereas the 'bilayer' width on graphite is $\sim 38 \text{ \AA}$ (Fig. 1*b*). The adsorbed molecules are bent in opposite directions in successive rows. From row to row there is a repeating periodicity of [(aromatic) head up/(aliphatic) tail down], [tail down/head up], [tail up/head down], [head down/tail up].

The packing arrangement is energetically unfavourable in terms of molecule-molecule interactions, because aromatic

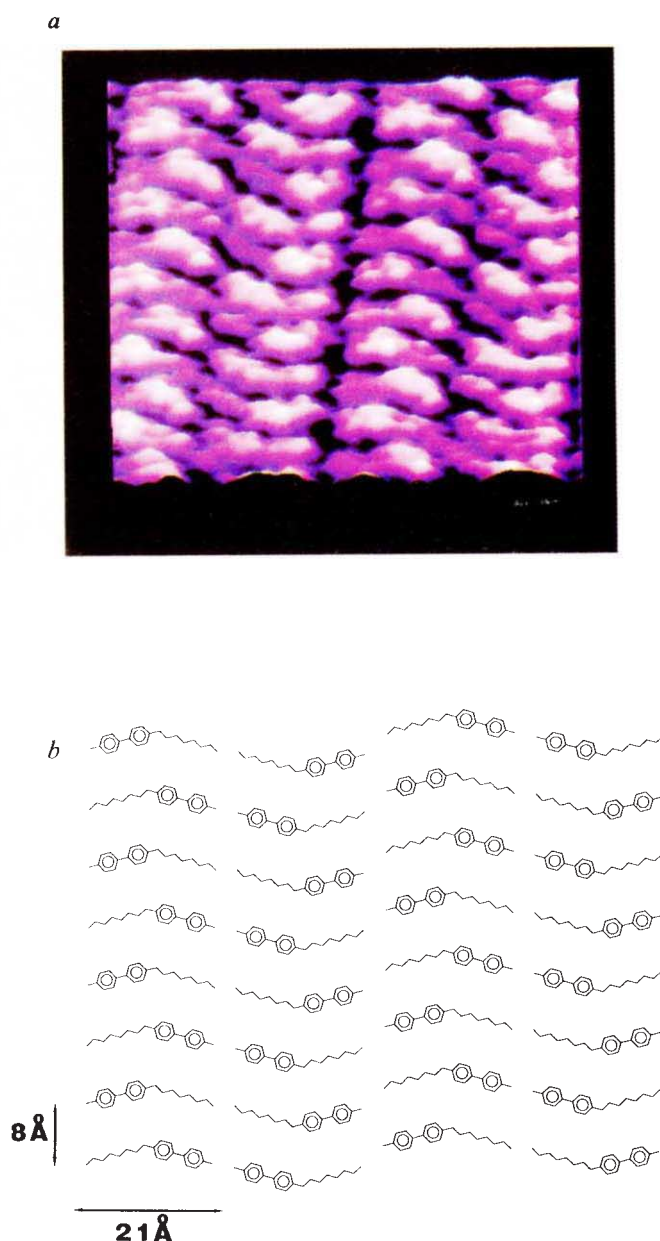


FIG. 2 *a*, STM image of 8CB on MoS_2 . Area of image is $80 \times 80 \text{ \AA}$. The alkyl tails are visible more clearly than in Fig. 1*a*. *b*, Model showing the packing of 8CB molecules on MoS_2 . The alkyl chains bend in opposite directions in neighbouring rows. The four-row periodicity (see text) was observed throughout the domain.

groups would prefer to be adjacent, while cyano dipoles would prefer to cancel one another. But the periodicities observed correlate with the lattice of the underlying MoS_2 substrate, implying strong molecule-surface interactions. The $8\text{-\AA}$ period between the molecules in a row is three times the MoS_2 lattice spacing ($2.7 \text{ \AA}$) or five times the periodicity ($1.6 \text{ \AA}$) perpendicular to this. Furthermore, the layer width of $21 \text{ \AA}$ corresponds to thirteen times $1.6 \text{ \AA}$. Thus the rows of 8CB molecules are probably aligned epitaxially on MoS_2 and are thereby anchored in an 'unfavourable' arrangement. We intend to further study the dependence of anchoring structures on the anchoring forces

by using other MX_2 as substrates.

Thus the packing of 8CB on MoS_2 reflects the underlying lattice structure in that the periodicities are integral multiples of the MoS_2 lattice spacings. Our results demonstrate that molecule-substrate interactions can control the structures of adsorbed liquid-crystal layers. An extensive discussion of the new periodic structure reported here, and possible anchoring mechanisms as well as higher modulation features, will be reported elsewhere. $\square$

Received 18 October 1989; accepted 24 January 1990.

1. Foster, J. S. & Frommer, J. E. *Nature* **333**, 542-545 (1988).
2. Foster, J. S., Frommer, J. E. & Spong, J. K. *Proc. SPIE, Liquid Crystal Chemistry, Physics and Applications* **1080**, 200-208 (1989).
3. Spong, J. K. *et al. Nature* **338**, 137-139 (1989).
4. Smith, D. P. E., Hörber, H., Gerber, Ch. & Binnig, G. *Science* **245**, 43-45 (1989).
5. Lindsay, S. M., Thundat, T., Nagahara, L., Knipping, U. & Rill, R. L. *Science* **244**, 1063-1064 (1989).
6. Arscott, P. G., Lee, G., Bloomfield, V. A. & Evans, D. F. *Nature* **339**, 484-486 (1989).
7. Sleator, T. & Tycko, R. *Phys. Rev. Lett.* **60**, 1418-1421 (1988).
8. Lippel, P. H., Wilson, R. J., Miller, M. D., Wöll, Ch. & Chiang, S. *Phys. Rev. Lett.* **62**, 171-174 (1989).
9. Leadbetter, A. J., Frost, J. C., Gaughan, J. P., Gray, G. W. & Mosley, A. *J. Phys., Paris* **40**, 375-380 (1979).
10. Hara, M., Sasabe, H., Yamada, A. & Garito, A. F. *Jap. J. appl. Phys.* **28**, L306-L308 (1989).

A composite construction material that solidifies in water

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A FLEXIBLE, waterproof material that will solidify in water has long been desired in civil engineering^{1,2}. We have developed a new class of material, called Aquaphalt, which has these and other desirable properties. Aquaphalt is composed of an asphalt emulsion, cement and a water-absorbing polymer. The components are liquid at ambient temperature and can therefore be pumped, but they form a gel almost instantly when mixed. The hardened mixture is similar to hard bitumen, and has very low water permeability, high ductility and good adhesion to other materials. Here we describe the characteristics of Aquaphalt, with particular emphasis on those properties that give it potential as a shock-absorbing, waterproof backfill material for tunnels and dams, and as an antiliquefaction agent for protection of buildings exposed to earthquake hazards.

Table 1 shows the compositions of several varieties of Aquaphalt. The basic material comprises bitumen emulsion (non-ionic)^{3,4}, cement and a high-water-absorbing polymer (accogel)⁵⁻⁸. Accogel is a liquid polymer emulsion in which the polymer consists of a cross-linked polymer prepared with cationic methacrylate polymer aggregated into particles of size 3-5 μm . It has a water-absorbing capacity of more than 300 times its initial volume in fresh water.

Figure 1 shows the results of a cone-penetration test (which measures reduced compressive strength, estimated by the volume of the indentation) and an axial compression test (compressive strength) conducted on Aquaphalt after 7 days of curing in water⁹. The reduced compressive strength of the mixtures that include jet cement (specimens 5 and 6) is ~ 0.05 MPa after 1 h and ~ 0.1 - 0.2 MPa after 7 days, whereas for the mixtures that use high early Portland cement the reduced compressive strength is < 0.002 after ~ 1 h yet again rises to ~ 0.1 MPa after 7 days. As shown in Fig. 1a, the strength of Aquaphalt after 1 day is relatively insensitive to the content of water-absorbing polymer, but it increases slightly with increasing cement content

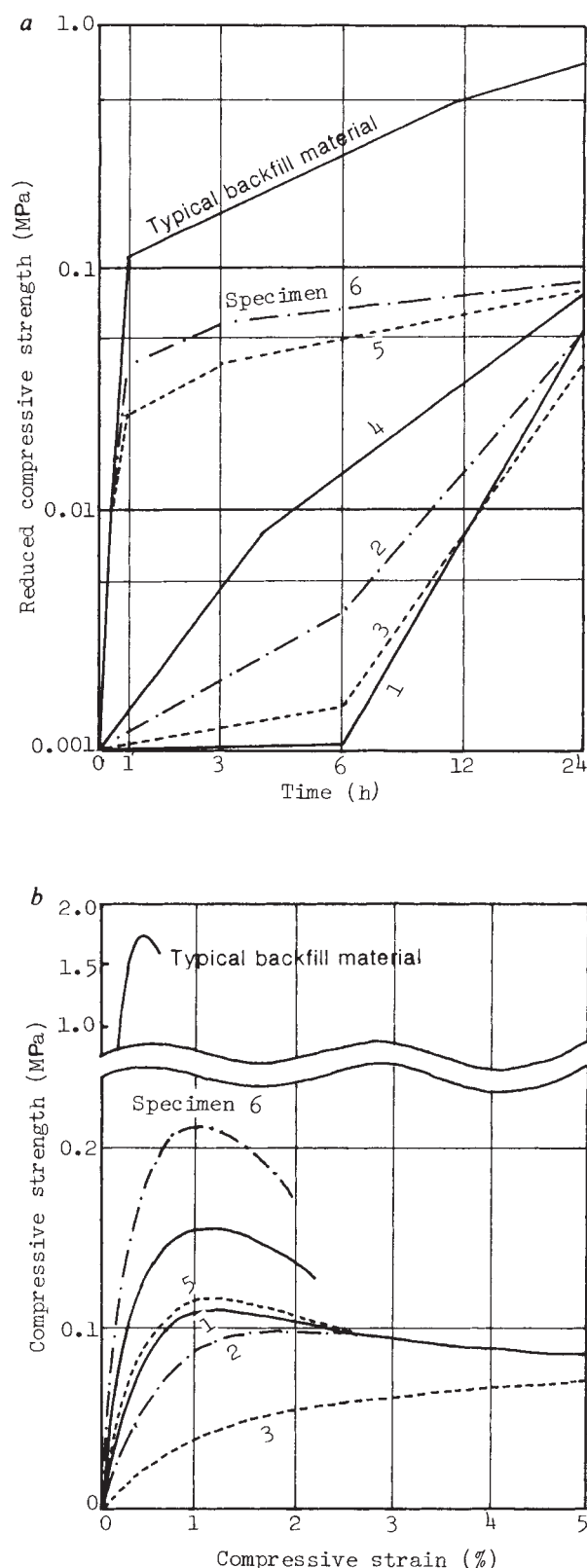


FIG. 1 *a*, Reduced compressive strength estimated by the volume of the indentation as a function of time. *b*, Compressive strength as a function of compressive strain. For *a*, the test method was the cone-penetration test. A cone made of steel (weight 0.1 kg, head angle 15° , 5 s) is allowed to penetrate into the material under its own weight at 15°C . The volume of the indentation is a measure of the material's reduced compressive strength. For *b*, the test method was the axial compression test, under which a cylindrical specimen (5 cm diameter and 10 cm height) at 15°C is compressed at a rate of strain of $1.0\% \text{ min}^{-1}$. Temperature was 15°C .

TABLE 1 Composition of Aquaphalt

Specimen	1	2	3	4	5	6
Asphalt emulsion (non-ionic)	2,000	2,000	2,000	2,000	2,000	2,000
High early Portland cement	600	600	600	800		
Jet cement					400	600
High-water-absorbing polymer (accogel)	40	60	80	60	60	40

Amounts are in g. Specimen 2 was used as the 'standard' sample for the tests in Fig. 2.

TABLE 2 Results of permeability test

Curing time in water	7 days			28 days		
Confining pressure (MPa)	0.1	0.3		0.1	0.3	0.6
Permeability ($\times 10^{-10} \text{ cm s}^{-1}$)	80	2.0–20		8.9–19	$\bar{x}_9 = 3.1^*$	$\bar{x}_6 = 3.3^*$
Aquaphalt (specimen 2)					$\sigma_9 = 1.4^\dagger$	$\sigma_6 = 1.9^\dagger$
Common plastic backfill material	1,700	2,200				

* Average.

† Standard deviation.

(specimen 4). For comparison, the compressive strength of the plastic material (TGS-II which mainly consists of cement, lime and fine slag) commonly used for backfill material is 0.1 MPa after one hour and 2 MPa at 7 days (see Fig. 1b).

The strength of Aquaphalt is thus much lower than that of materials commonly used as backfill in, say, tunnels. On the other hand, ductility is much higher for Aquaphalt. Ductility is in fact the more important property for such applications because, for example, earthquakes can cause movement of surrounding rock sufficient to fracture normal backfill material (values of the strain at failure are typically $\sim 0.5\%$); this can lead to fracture of tunnel segments themselves, creating leaks. Aquaphalt, however, fails at 1%–5% strain, making it ductile enough to cushion the shock from earthquakes.

Split-cylindrical shear tests were conducted using specimen 2. Cylindrical concrete blocks (diameter 100 mm, height 60 mm) were cut along the axis and Aquaphalt was poured (in air) to a thickness of 3.7 mm between the two half-cylinders. The shear test was conducted at a strain rate of $4.5 \times 10^{-3} \text{ s}^{-1}$ and a temperature of 15°C . The shear strength was measured as 0.05 MPa, and the strain at maximum load was 0.57. Aquaphalt shows good adhesion to concrete—fracture into two pieces occurred at the Aquaphalt–concrete junction only at a strain of 5.4.

Table 2 shows the results of the permeability test. Aquaphalt is highly waterproof (comparable with cement), with a water permeability of 10^{-9} – $10^{-10} \text{ cm s}^{-1}$ under 0.1–0.6 MPa pressure. Like bitumen, the permeability drops slightly under high confining pressure.

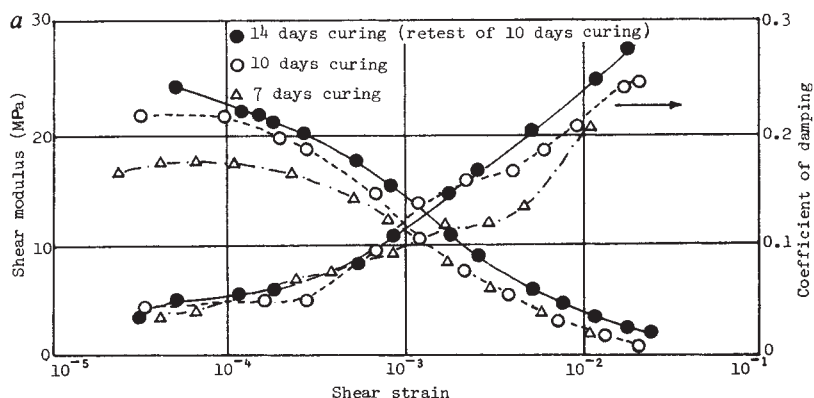


FIG. 2 a, Shear modulus and damping coefficient as a function of strain. The test method was the hollow-cylindrical torsion test. A hollow cylinder of Aquaphalt (outer diameter 10 cm, inner diameter 6 cm, height 17.5 cm), cured for 7–14 days in water at 20°C , is fixed at both ends and subjected to a torque. Stress is controlled by an electronic servo under the following conditions: confining pressure, 0.04 MPa; frequency of sinusoidal wave, 0.05 Hz; temperature, 20°C ; maximum stress, 0.1 MPa. b, Stress–strain hysteresis curve of Aquaphalt. Specimens of mixture 2 (cured for 10 days) were subjected to an alternating stress of 0.03 MPa at 20°C , resulting in an initial strain of 1%.

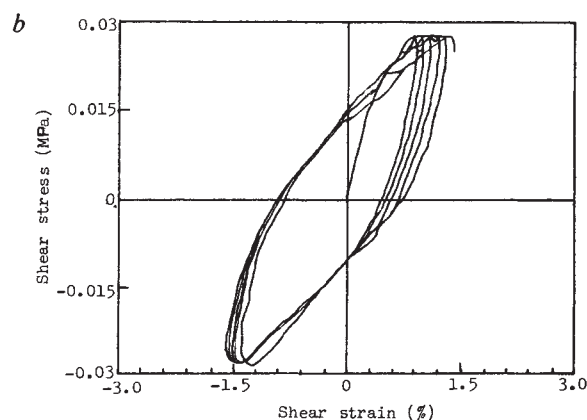


Figure 2 shows the results of the torsion (shear-modulus) test on a cylindrical hollow specimen. The shear modulus is ~ 20 MPa at a strain of 1×10^{-4} . In Fig. 2a, the open circles show the shear modulus of specimen 2 after 10 days curing in water under a stress of 0.04 MPa. These same samples were then reimmersed in water for 4 days without an applied stress, and were then retested (filled circles in Fig. 2a). Materials usually show a greatly reduced shear modulus in the second test because of microscopic fracture incurred during the first. In this case, however, shear modulus—and thus strength—were not affected adversely. The damping coefficient (which characterizes the ability to absorb external energy) is significantly higher than that of present backfill materials. Similar results were obtained for the other mixtures.

Figure 2b shows the stress-strain hysteresis curve of Aquaphalt. Normal backfill materials fracture at these levels of strain, but Aquaphalt withstood five loading cycles.

Buildings constructed on sandy ground are particularly prone to earthquake hazards because vibration can cause liquefaction of the sand. Such buildings must therefore be supported by deep pilings which penetrate into the bedrock below; this construction design is costly. Aquaphalt could be used as a foundation material in sandy soil, where it would have two functions. First, it would stiffen the soil, reducing the risk of liquefaction and assisting the support of the building; second, the hysteresis characteristics would enable it to absorb some of the energy of the earthquakes, thereby reducing structural stresses.

Aquaphalt also has attractive possibilities as a backfill material in tunnel construction. Tunnels are typically inserted in segments behind a boring machine that creates the initial passage. A narrow space exists between the segment and the rock face, which is filled with the backfill material. This is injected into the space when all the segments are in place. Commonly used backfill materials have a viscosity of $\sim 6,000$ – $7,000$ N s m $^{-2}$ at the moment of injection; but this increases rapidly with time, so that the materials do not spread well, leaving voids that allow water from surrounding rock to leak through the cracks between segments.

Aquaphalt, however, could be transported to the site in the form of two liquids: a mixture of the bitumen emulsion (viscosity of 30 N s m $^{-2}$ at 15 °C) and cement (with a combined viscosity of ~ 90 N s m $^{-2}$ at 15 °C for 30% cement), and the water-absorbing polymer (viscosity of 50–100 N s m $^{-2}$ at 15 °C). These two liquids can then be mixed *in situ* before injection; the time required for mixing is adjustable by varying the liquid compositions. The viscosity is typically 7,000 N s m $^{-2}$ immediately after mixing at 15 °C, but increases to only 10,000 N s m $^{-2}$ 60 s later, which is, markedly lower than that of standard backfill material. Subsequent injection of this low-viscosity gel into the space between the segment and rock face fills it completely. Unlike bitumen emulsion, the gel does not disperse in water but repels it, pushing it back into cracks in the rock face or through drainage ports in the tunnel segments. Adhesion to the rock and segments is good, and as the gel solidifies over several days it swells slightly, maintaining the seal between the tunnel and the rock face.

Aquaphalt is especially suitable as a backfill material in tunnels located in soft ground under high water pressure. An example is the Tokyo Bay Tunnel, which is to commence construction soon; Aquaphalt is being examined for this application. □

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1. Nakahara, Y. et al. *A. Rep. Kajima Inst. Construction Technol. Kajima Corp.* Vol. 29 (June) 1–8 (1981) (in Japanese).
2. Soast, A. *Engng News Record* July 20, 26–28 (1989).
3. *Asphalt Emulsion Catalogue* (Toa douru kougyo Co. Ltd, Tokyo, 1987).
4. Inagaki, K. *Yukagaku* **18**(9), 566–573 (1969) (in Japanese).
5. Japan Patent 63-232888 (Mitsui Cyanamid Co. Ltd, 1988).
6. Japan Patent 63-312421 (Mitsui Cyanamid Co. Ltd, 1988).
7. Japan Patent 1-235794 (Mitsui Cyanamid Co. Ltd, 1989).
8. Watanabe, N. *Jap. J. Paper Technol.* **31**(7), 33–37 (1988) (in Japanese).
9. Alexander, P. & Blott, J. F. *J. Soc. Chem. Ind.* **64**, 14–15 (1945).

Crystallization and emplacement chronology of the Fournier oceanic fragment, Canadian Appalachians

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COLLISION zones between the plates that form the Earth's surface are commonly delineated by displaced fragments of oceanic crust and mantle. These fragments are important because they can be used to constrain the timing of the generation and destruction of former oceans by means of radiometric and fossil studies. In the Northern Appalachian/Caledonian orogen of Canada, the United Kingdom and Scandinavia, the majority of the oceanic fragments crystallized between 480 and 500 Myr ago in basins marginal to the Iapetus Ocean, and were emplaced onto continental margins at least 10 Myr later. Here we report a significant exception: U/Pb geochronology of zircons extracted from a plagiogranite in the Fournier oceanic fragment of the Canadian Appalachians reveals a much younger crystallization age of 461 ± 3 Myr, whereas fossil evidence indicates continental incorporation in the Silurian. Some Iapetan marginal basins therefore continued to develop, or were created, after the early Ordovician.

Establishing the age and mode of generation and emplacement of fragments of oceanic lithosphere preserved within the Appalachian/Caledonian orogen is critical for understanding the demise of the Iapetus Ocean and the ensuing collision between the Laurentian and Baltic plates during the Palaeozoic. Pertaining to this, a discontinuous chain of ultrabasic–basic bodies, interpreted to be fragments of displaced oceanic lithosphere, occurs in the Canadian Appalachians stretching from southern Quebec through New Brunswick to Newfoundland (Fig. 1a). This chain occurs near to the so-called Baie Verte–Brompton line¹ which, before the opening of the Atlantic Ocean in the Mesozoic, may have been contiguous with parts of what are now Ireland and Scotland in a zone whose northern boundary includes oceanic fragments associated with the Omagh² and Highland Boundary Faults^{3,4}. The exact nature of the Baie Verte–Brompton line is debatable, but it appears to be a major suture in the form of a fault complex that has undergone thrusting and transcurrent movement at different stages of its evolution⁵. This also agrees with interpretations of the structural history of the Highland Boundary Fault in Scotland⁶. The oceanic fragments are not restricted to the Baie Verte–Brompton line/Highland Boundary Fault: some occur to the west of it (for example, Bay of Islands, Newfoundland⁷ and Shetland, north-east Scotland⁸) and some to the east (for example, Coy Pond and Pipestone Pond, Newfoundland⁹ and Ballantrae, southwest Scotland¹⁰), such that they define a corridor at least 200 km wide. Many of the studied oceanic fragments in Canada, the United Kingdom and Scandinavia have yielded primary ages between ~ 480 – 500 Myr (namely, early Ordovician) and possess geochemical affinities indicative of marginal basin origins^{8,9,11,12}. Until now, the Fournier oceanic fragment has received relatively little documentation, yet potentially it provides a valuable additional piece of the Appalachian/Caledonian jigsaw puzzle.

The Fournier oceanic fragment is exposed along the Baie des Chaleurs coast in northern New Brunswick and outcrops inland over an area of ~ 60 km² (Fig. 1b). It comprises several intrusive generations of massive, clinopyroxene–plagioclase gabbro (with a combined thickness of at least 1 km) which, in its upper levels, gives way to a clinopyroxene–plagioclase dyke complex (~ 1 km

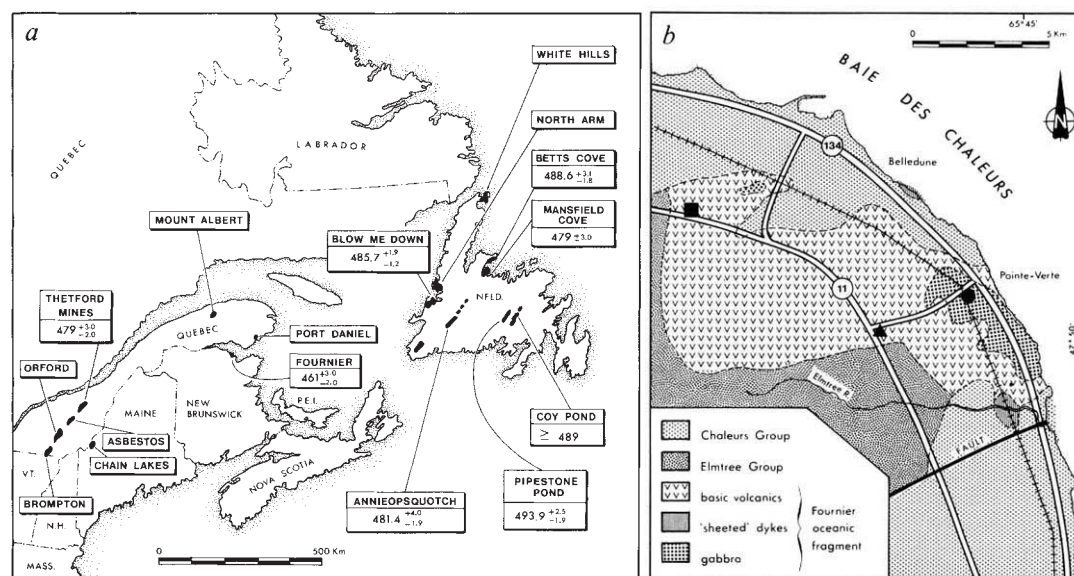


FIG. 1 a, Occurrences of displaced oceanic fragments within the Canadian Appalachians and western Maine. Selected primary age data are shown in boxes. All are U/Pb dates obtained from zircons extracted from plagiogranites and, with the exceptions of the ages for the Fournier oceanic fragment (this work), Thetford Mines¹¹ and Mansfield Cove²⁸, were obtained by G. R. D. and T. E. Krogh⁹. b, Simplified geology of the Fournier oceanic fragment of northern New Brunswick. Square, graptolite locality²³; triangle, conodont

locality²⁴; circle, dated plagiogranite locality at Pointe-Verte quarry (see Fig. 2 and Table 1 for results). The Elmtree Group is late Ordovician to early Silurian in age, comprises a lower unit basalt with minor felsic volcanics and a sedimentary upper unit and appears to conformably overlie the Fournier oceanic fragment³¹. The Chaleurs Group is wholly sedimentary and is of Silurian age²⁵.

thick) that is locally sheeted (for example, along the coast southeast of Belledune, Fig. 1b). A melange unit, ~0.5 km thick, comprising greywacke, slate and chert, with olistoliths of pillow lava (~5 m diameter), separates the predominantly basic intrusive components of the complex from a pillow lava unit (~2.5 km thick). Sediments within and overlying the pillow lavas include graptolite-bearing grey shales, conodont-bearing interpillow carbonates, 'ophiolitic' sandstones and red siliceous mudstones.

The whole sequence has been variably metamorphosed. The gabbros are pervaded by discrete upper amphibolite facies shear zones (0.5–2 m wide) that are commonly found closely associated with deformed and undeformed plagiogranite veins and pods (quartz + albite ± allanite), although in places plagiogranite clearly cuts discordantly into the host gabbro (for instance, at Pointe-Verte quarry, Fig. 1b). Away from the high grade shear zones, the massive gabbros show patchy, greenschist facies static overprinting with the development of chlorite, actinolitic hornblende, epidote and albite. The basic dykes show similar overprinting, but are more pervasively altered. The pillow lavas show static prehnite–pumpellyite facies overprinting with the development of prehnite, pumpellyite, epidote and chlorite, and zeolite and carbonate amygdalae.

Ultrabasic rocks have not been found in direct association with the Fournier lithologies, but screens of serpentinized harzburgite occur in fault zones associated with the Rocky-Brook Millstream Fault in the Alcida area to the southwest of the Elmtree River¹³ and as pebbles and boulders in the Armstrong Brook Formation of the overlying Chaleurs Group (J. P. A. Noble, personal communication).

The oceanic affinities of the Fournier lithologies are clearly indicated by the presence of pillow lavas, various marine sediments, including cherts and other pelagics, and the nearby occurrence of serpentinites. In this respect, the complex complies with the definition of a Steinmann Trinity¹⁴. The style of metamorphic overprinting is also typical of that found in ocean crust, with a progressive increase in grade from low to higher facies when moving down sequence: an effect that can be attributed to sub-sea floor hydrothermal circulation in the vicinity of a spreading centre. Previous workers have referred to the Fournier oceanic fragment as an ophiolite¹⁵, even though not all the components necessary to comply with the Penrose definition are present¹⁶.

Major-, trace- and rare-earth element chemistry of the Fournier lithologies show a mid-ocean-ridge basalt (MORB)

TABLE 1 U/Pb isotope data obtained from zircons separated from the plagiogranite of Pointe-verte quarry (see Fig. 1b for location)

Fractions	Concentrations measured				Atomic ratios [‡]					Age (Myr)
	Weight* (mg)	U (p.p.m.)	Pb (radiogenic)	Total common Pb (pg)	$\frac{^{206}\text{Pb}}{^{204}\text{Pb}}$ [†]	$\frac{^{208}\text{Pb}}{^{206}\text{Pb}}$	$\frac{^{206}\text{Pb}}{^{238}\text{U}}$	$\frac{^{207}\text{Pb}}{^{235}\text{U}}$	$\frac{^{207}\text{Pb}}{^{206}\text{Pb}}$	
+100 clear fragments, abraded	0.093	204	17.1	13	6,942	0.257	0.07399 ± 26	0.5735 ± 21	0.05621 ± 10	461
+70 clear euhedra, abraded	0.240	223	18.5	8	29,572	0.254	0.07340 ± 30	0.5696 ± 24	0.05628 ± 7	463

* Uncertainty in weight ±0.003 mg (1σ).

† Corrected for fractionation and common Pb in the spike.

‡ Corrected for fractionation and spike, 5–10 pg Pb laboratory blank and initial common lead calculated from the model of Stacey and Kramers³⁰ and 2 pg U blank.

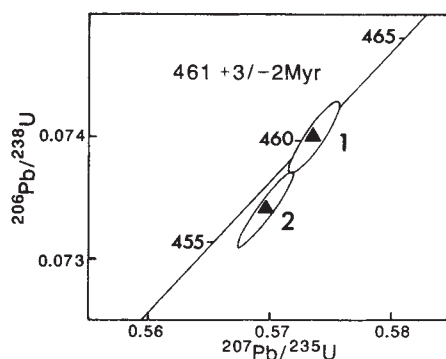


FIG. 2 U/Pb concordia diagram showing data obtained from zircons separated from the plagiogranite of Pointe-Verte quarry (see Fig. 1b for location).

signature predominating, although some of the pillow lavas and dykes possess island arc affinities¹⁷. All lithologies, with the exception of some of the gabbros, indicate derivation from a mantle source enriched in light rare-earth elements and, overall, the evidence suggests that the oceanic fragment crystallized in a supra-subduction zone setting¹² (in a marginal oceanic basin undergoing incipient arc development above a downgoing lithospheric slab).

To constrain the primary crystallization age of the Fournier oceanic fragment, zircons were separated from a plagiogranite sample obtained from Pointe-Verte quarry (see Fig. 1b for location). The zircons were extracted by standard techniques of crushing and mineral separation using heavy liquids and a Frantz Isodynamic separator. The zircons were coarse-grained and of high quality and included both angular fragments and euhedral grains. One fraction of each was hand picked and abraded¹⁸. Dissolution and chemical separation followed the technique of Krogh¹⁹ and uncertainties in the isotopic ratios were calculated according to the procedure of Ludwig²⁰. These are reported at the 2 σ level in Table 1. The analysis of the coarse fragments is concordant with a $^{207}\text{Pb}/^{206}\text{Pb}$ age of 461 Myr, whereas the euhedral grains are 1.3% discordant with an age of 463 Myr (Fig. 2). These overlap within measurement uncertainties yielding 461 ± 3 Myr as the age of crystallization of the plagiogranite (namely, Llandeilo).

Although some workers have shown that certain Appalachian/Caledonian plagiogranites have been intruded after the formation of oceanic crust due to subsequent arc-related plutonism¹¹ or off-axis igneous activity²¹, the plagiogranites of the Fournier oceanic fragment are clearly syn-magmatic with respect to oceanic crust formation. This is proved by their occurrence in high-temperature amphibolitic shear zones and in plastically deformed but otherwise unaltered gabbros, both of which require temperatures $>600^\circ\text{C}$ for their formation. Such conditions can only be realized near to a hotspot (for example, a spreading centre). The observation that the plagiogranites occur pre-, syn- and immediately post- the high-temperature shear zones is further evidence of their near-ridge syn-magmatic/syn-deformation origins. This interpretation is supported independently by a K/Ar age of 462 ± 23 Myr obtained from an amphibole separate in the same shear zone from which our plagiogranite sample was taken²² and a U/Pb estimated age of 461 Myr recently obtained from zircons extracted from the adjacent host gabbro²¹. Fossil evidence in support of the radiometric data is provided by graptolites obtained from black slate interbedded with the pillow lavas²³ and conodonts obtained from interpillow limestone²⁴, both of which indicate a Llandeilo to early Caradoc age (see Fig. 1b for sample locations).

Establishing the timing of the continental incorporation of the Fournier oceanic fragment is more problematic. One critical constraint is the presence of the Armstrong Brook Formation at the base of the Chaleurs Group which unconformably overlies the Fournier package²⁵ (Fig. 1b). This comprises pebbles and boulders of pillow lava, dolerite, gabbro, amphibolite and peridotite derived from the oceanic fragment, as well as quartz and

jasper, in a matrix of grits, sandstones and shales containing brachiopod fauna of Llandovery C age²⁵. The Armstrong Brook Formation is of predominantly fluvial origin and was generated by the rapid erosion of a terrain with high topographic relief that was subaerially exposed²⁵. This indicates that the oceanic fragment was elevated (overthrust) by the beginning of the Silurian and that its continental emplacement/docking took place no later than Llandovery times. In the absence of any sedimentary evidence supporting an earlier emplacement age, the Llandovery Armstrong Brook Formation is taken to indicate the timing of continental incorporation of the Fournier oceanic fragment. This suggests that a period of at least 20 Myr elapsed between oceanic crystallization and final continental emplacement (see, for example, ref. 26).

Metamorphic and tectonic overprinting of the Fournier oceanic fragment, due to syn- to post-emplacement continental orogenesis, arose due to the emplacement of the fragment by means of low-angle thrusts (late Taconic), followed by low greenschist facies overprinting combined with open folding about northeast-southwest axes and regional cleavage development (Acadian)²⁷. These events contributed to the retrogression of the higher-grade, near-ridge oceanic metamorphic effects and the overall dismembering of the fragment.

The 461 ± 3 Myr primary age for the Fournier oceanic fragment is significant because it is younger than most of the other oceanic fragments of the Northern Appalachian/Caledonian orogen. Most of the fragments of Newfoundland^{9,28}, Ireland², Scotland²⁹ and Scandinavia¹¹ have ages between 480–500 Myr indicating early Ordovician generation within marginal oceanic basins. This implies that either a distinct marginal basin succeeded the existence of the more major back-arc basin(s) from which the majority of the Appalachian/Caledonian oceanic fragments were derived or, parts of an earlier marginal basin continued to develop into the middle Ordovician. Evidence for the Fournier oceanic fragment not being an isolated example of later marginal basin development is afforded by the Solund oceanic fragment of Norway: U/Pb zircon data from this body reveals a crystallization age of 443 ± 3 Myr¹¹. Clearly, more geochemical and primary age data are required for the oceanic fragments of the Appalachian/Caledonian orogen to substantiate the existence of distinct marginal basins throughout the Ordovician. □

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- Williams, H. *Can. J. Earth Sci.* **21**, 887–901 (1984).
- Hutton, D. H. W., Aftalion, M. & Halliday, A. N. *Nature* **315**, 210–212 (1985).
- Henderson, W. G. & Robertson, A. H. F. *J. geol. Soc. Lond.* **139**, 433–450 (1982).
- Ikin, N. P. *J. geol. Soc. Lond.* **140**, 267–268 (1983).
- Goodwin, L. B. & Williams, P. F. *Eos* **70**, 1311 (1989).
- Hutton, D. H. W. *Geol. Mag.* **124**, 405–425 (1987).
- Church, W. R. & Stevens, R. K. *J. geophys. Res.* **76**, 1460–1466 (1971).
- Spray, J. G. *Can. J. Earth Sci.* **25**, 310–325 (1988).
- Dunning, G. R. & Krogh, T. E. *Can. J. Earth Sci.* **22**, 1659–1670 (1985).
- Spray, J. G. & Williams, G. D. *J. geol. Soc. Lond.* **137**, 359–368 (1980).
- Dunning, G. R. & Pedersen, R. B. *Contr. Miner. Petrol.* **98**, 13–23 (1988).
- Pearce, J. A., Lippard, S. J. & Roberts, S. *Spec. Publ. geol. Soc. Lond.* **16**, 77–94 (1984).
- Paktunc, A. D. & Ketchum, J. W. F. *Pap. geol. Surv. Can.* **69-18**, 15–22 (1989).
- Steinmann, G. *Int. Geol. Congr. Madrid* **2**, 638–667 (1927).
- Rast, N. & Stringer, P. *Tectonophysics* **69**, 221–245 (1980).
- Geotimes* **17**, 24–25 (1972).

17. Flagler, P. A. thesis, Univ. New Brunswick (1989).
18. Krogh, T. E. *Geochim. cosmochim. Acta* **37**, 485-494 (1973).
19. Krogh, T. E. *Geochim. cosmochim. Acta* **46**, 637-649 (1982).
20. Ludwig, K. R. *Earth planet. Sci. Lett.* **46**, 212-220 (1980).
21. van Staal, C. R., Langton, J. P. & Sullivan, R. W. *Pap. geol. Surv. Can.* **88-2**, 37-40 (1988).
22. Fyffe, L. R. & Davies, J. L. *Fredericton '85 Field Excursion Vol. 2*, 40-48 (University of New Brunswick Press, Fredericton, 1985).
23. Fyffe, L. R. 11th Ann. Rep. New Brunswick Dept Nat. Res. Energy 43-45 (1986).
24. Nowlan, G. *Geol. Surv. Can. Rep.* 005-GSN (1986).
25. Noble, J. P. A. *Can. J. Earth Sci.* **13**, 537-546 (1976).
26. Spray, J. G. & Roddick, J. C. *Contr. Miner. Petrol.* **72**, 43-55 (1980).
27. Stringer, P. *Can. J. Earth Sci.* **12**, 949-961 (1975).
28. Dunning, G. R. *et al. Can. J. Earth Sci.* **24**, 1175-1184 (1987).
29. Bluck, B. J. *et al. Geology* **8**, 492-495 (1980).
30. Stacey, J. S. & Kramers, J. D. *Earth planet. Sci. Lett.* **26**, 207-221 (1975).
31. Williams, G. L. *et al. Can. Soc. Petrol. Geol. Vol. 6* (1985).

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Decoupling of chemical and isotopic exchange during magma mixing

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WHEN silicate magmas of different chemical and isotopic compositions are brought into contact, their equilibration is governed by the kinetics of diffusion. Here I present experimental data showing that, between a basic and an acidic silicate melt, isotopic exchange proceeds much more quickly than chemical homogenization. Moreover, different isotopic systems (in this case, strontium and neodymium) exhibit different rates of equilibration. The transient compositional states reached in the exchange process depart markedly from the results expected for binary mixing of the two end-members¹. This complexity must be acknowledged in efforts to unravel episodes of magma mixing, such as involving partial melting of (acidic) continental crust by basic magma. In such cases, the intimate mixing of chemically dissimilar magmas is hindered by their different viscosities, but they do selectively exchange mass by diffusion.

Liquid-state interdiffusion is studied in the laboratory using natural rock powders of unaltered dolerite from the East Greenland macrodiike complex and Precambrian leucogneiss representing the host rock for these mafic intrusions². The crustal signature of the felsic reservoir is magnified by doping the leucogneiss (sample FS-R) with ⁸⁷Sr-enriched SrCO₃ and ¹⁴⁴Nd-enriched NdO. The mafic reservoir (sample MF-R) is similarly doped with reagent grade SrCO₃ and NdO to restore the relative abundance levels of Sr and Nd found in the natural rocks. The overall concentrations of these elements are 30-150 times those found in natural materials. Compositions of the starting materials are shown in Table 1. Diffusion couples, ~2.5-mm long interact for a prescribed time under isothermal conditions at 10 kbar in the single-stage 0.5-inch diameter piston-cylinder device and are quenched to glass. Major-element gradients within the interfacial region of the reservoirs are determined using an electron microprobe. Variations in Sr and Nd concentration and isotopic composition across the same region are measured by ion microprobe (see Table 1).

Results for two experiments are presented for comparison in Fig. 1. The diffusion profiles developed in these experiments are distinctive and display a number of important features of the homogenization process. For the 1.5 h/1,255 °C run, the silica gradient, as well as the profiles of the ⁸⁷Sr/⁸⁶Sr and ¹⁴³Nd/¹⁴⁴Nd ratios, vary smoothly across the interfacial region of the couple and asymptotically approach the initial compositions of reservoirs at the distal ends of the charge. Some degree of asymmetry is exhibited by each of these profiles (greatest for ¹⁴³Nd/¹⁴⁴Nd), indicating that the diffusivities governing

exchange depend on bulk composition (see Fig. 1). In marked contrast, the concentration profiles for Sr and Nd show pronounced maxima and minima within the interfacial regions of the couple. These latter features are indicative of 'uphill' diffusion, presumably due to strong coupling of the chemical fluxes of these components to those of the major melt

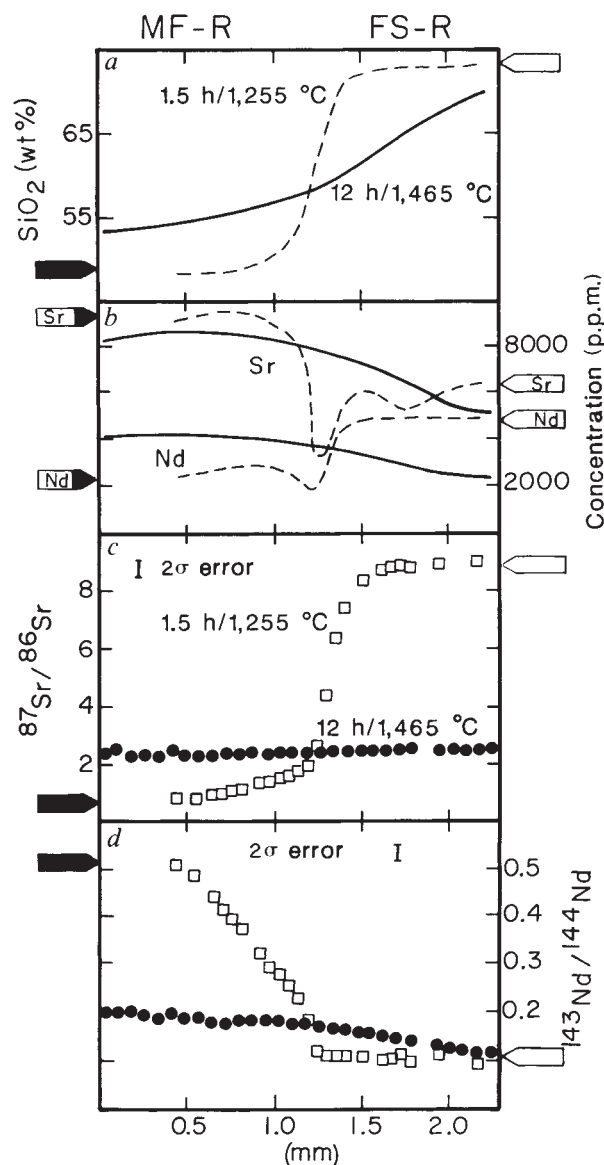


FIG. 1 Chemical and isotopic variations measured normal to the original mated interface of the experimental interdiffusion couples as determined by electron and ion microprobes. Results shown for 10-kbar experiments performed in the 0.5 inch piston-cylinder apparatus at 1,255 °C for 1.5 h (dashed lines and open symbols) and 1,465 °C for 12 h (bold lines and filled symbols). The silica content (in wt%) plotted as a function of distance (in millimetres) across the experimental couples is shown in *a*. *b*, Variations in the concentrations of Sr and Nd in p.p.m. are shown for the same couples, whereas the corresponding variations in Sr and Nd isotopic ratios are shown in *c* and *d*. Error bars for ⁸⁷Sr/⁸⁶Sr and ¹⁴³Nd/¹⁴⁴Nd ratios represent the average precision for each value at the 95% confidence level as obtained from the Massachusetts Institute of Technology/Harvard/Brown Cameca IMS 3f ion microprobe. The initial compositions of the mafic (MF-R) and felsic (FS-R) reservoirs in both interdiffusion couples are indicated by bold and open arrows, respectively. A Boltzmann-Matano (BM) analysis²² of interdiffusion profiles of the 1.5 h/1,255 °C experiment gives a diffusivity of $1.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-2}$ for Sr isotopic exchange in the mafic portion of the couple (50 wt % SiO₂). The value decreases with increasing silica content and at 70 wt % SiO₂ is $5.1 \times 10^{-8} \text{ cm}^2 \text{ s}^{-2}$. This range of values is a factor of two greater than that determined for Nd isotopic exchange and exceeds the rate of chemical homogenization (based on a BM analysis of the silica gradient) by a factor of 5-7 (ref. 23).

constituents (most notably SiO_2), as has been previously noted for the alkali and certain transition metal cations³⁻⁵. What is perhaps most surprising is the lack of correlated Sr and Nd chemical and isotopic exchange. Strong 'uphill' diffusive effects seen in the chemical profiles are not evident in the isotopic ratio profiles.

Further evidence of decoupling of chemical and isotopic exchange is found in the 12 h/1,465 °C experiment, shown in Fig. 1. Although diffusion has modified the composition of liquids at the extreme ends of the charge, large chemical gradients persist. Most notably Nd, which was initially enriched in the felsic reservoir, is found to concentrate in the silica-poor region of the charge. Sr also is enriched at this end, but shows no irregularities indicative of 'uphill' diffusion. These observations alone indicate extensive transport of Sr and Nd, but in a manner that has not resulted in chemical homogenization. The persistence of chemical gradients in the 12-h run is even more remarkable given that Sr isotopic homogeneity has been attained. In contrast, the Nd isotope gradient has diminished by 75%, whereas the silica gradient has decayed by only 36%. Clearly, the rates of isotopic and chemical exchange must differ to account for these relationships. Moreover, to account for differences in the degree of Sr and Nd isotopic homogeneity, the rate of exchange of Sr isotopes must exceed that for Nd isotopes. Values of diffusive mixing rates determined for the 1.5 h/1,255 °C experiment are reported in the Fig. 1 legend.

Understanding why the rates for chemical and isotopic exchange should differ during diffusive mixing of molten basalt and rhyolite requires some appreciation of the structural reorganization that may be a consequence of a material flux. Isotopic homogenization may occur by exchange of isotopes between already-existing structural positions without reorganization of the diffusion medium. Chemical homogenization, however, may require reorganization of the structure of the diffusion medium to accommodate changes in the proportion of the chemical ingredients. This reorganization may be a less probable process than simple exchange of material on pre-existing sites. For example, silicate liquids have a short-range structural order determined by the tendency of SiO_4 and M^+AlO_4 tetrahedra to co-polymerize. Many experimental studies have shown that the mixing and unmixing of silicate liquids is largely regulated by the mobility of these network-forming ingredients³⁻⁸. Apparently, the activation for jumping and finding suitable landing sites for cumbersome polymer species is less probable than the activation required to move non-polymerizing ingredients⁸. Thus, easily activated network modifiers, such as Sr and Nd, have opportunities to exchange positions among themselves (that is, random walk) on timescales which are short compared to the formation of 'new' structural positions that allow for changes in the proportion of network formers to network modifiers. The fact that the rates of isotopic exchange for Sr and Nd differ from one another, as demonstrated in Fig. 1, relate to more subtle differences in bond energies and coordination of these network-modifying cations to oxygen⁹.

The mixing of magmas in chambers or conduits is a transient process involving the mechanical stirring of two or more fluids. Deformation and shearing result in an increase in interfacial area between contacting fluids and a reduction in the length scale of compositional heterogeneity. But, to alter the composition of intermingled magmas, material must be transferred across mutual boundaries by diffusion. As molecular diffusivities are small (10^{-6} – 10^{-8} $\text{cm}^2 \text{s}^{-1}$) for silicate liquids⁹, the length scales on which compositional heterogeneities can exist must also be small (metres or less) to produce homogenized products on reasonable convective timescales (10^3 – 10^4 yr)¹⁰. This fact alone suggests that only under ideal conditions will mixing go to completion before magmas erupt or crystallize. Furthermore, even after magmas come into physical contact differences in rheology and density give rise to mechanical forces that promote segregation¹⁰⁻¹³. The geochemical ramifications for mixing

events that do not lead to bulk homogenization are poorly understood, although there is abundant evidence for 'incompletely' mixed magmas from volcanic and plutonic environments^{2,14-16}. This raises the obvious question: how do hybrid rocks produced during the transient stages of mixing relate to one another and to the end-member compositions from which they evolve?

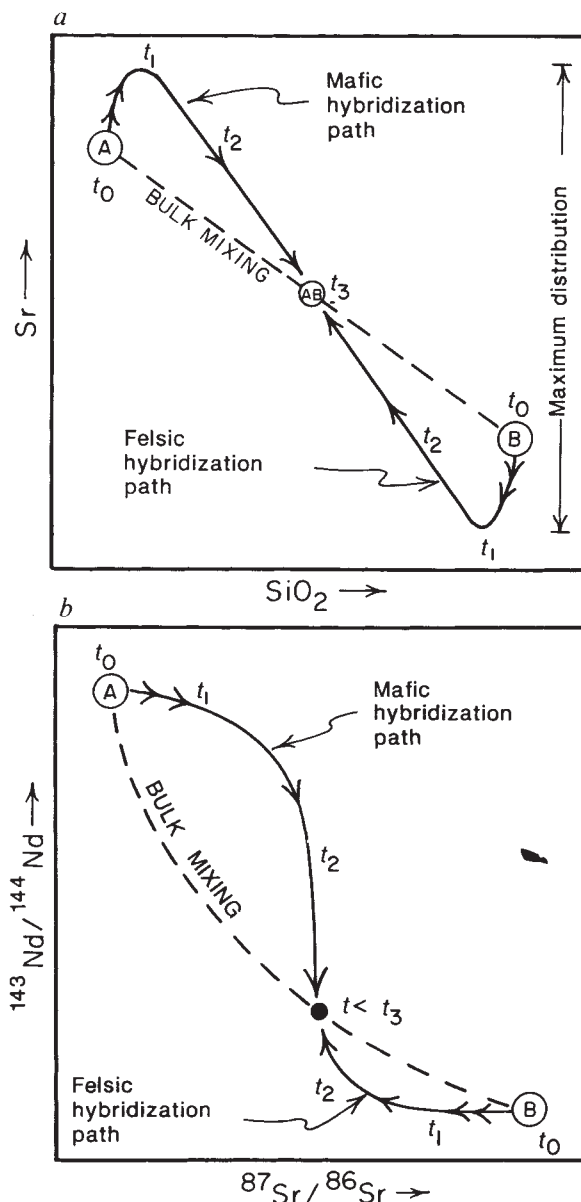


FIG. 2 Comparison of progressive hybridization of intermingled mafic (A) and felsic (B) magmas governed by interdiffusion with simple binary mixing. Solid curves portray irreversible hybridization paths for mafic (upper trajectories) and complementary felsic (lower trajectories) magmas leading to bulk homogenization. Dashed curves depict the full range of magma compositions produced by simple binary mixing involving end-members A and B. If mass is conserved for the system as a whole, but interdiffusion is permitted between intermingled parcels, complementary mixing paths converge with time to equivalent positions on the bulk-mixing hyperbola dictated by the initial volume proportions of the end-members. In a an initial stage of Sr 'uphill' diffusion ($t_0 \rightarrow t_1$) is indicated which minimizes Sr activity differences between magmas A and B. Further exchange, leading to complete homogenization (that is, $t_1 \rightarrow t_3$), is governed by bulk interdiffusion and rate-limited by silica diffusivity. In b covariations of $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are shown for magmas A and B when the rate of exchange of Sr isotopes exceeds that for Nd isotopes, as demonstrated by experiment. Isotopic equilibrium is achieved in time t_1 which is less than that required for the system to achieve chemical equilibrium (t_3) as shown in a.

TABLE 1 Natural starting materials for interdiffusion experiments

	MF-R	FS-R
SiO ₂	48.8	73.2
Al ₂ O ₃	13.6	14.2
TiO ₂	3.1	0.21
Fe ₂ O ₃	13.8	2.4
MgO	7.0	0.47
CaO	10.7	2.1
Na ₂ O	2.5	4.3
K ₂ O	0.34	3.1
Sr (p.p.m.)	9,250 ± 218	6,300 ± 104
⁸⁷ Sr/ ⁸⁶ Sr	0.708 ± 14	8.882 ± 177
Nd (p.p.m.)	2,110 ± 156	4,825 ± 207
¹⁴³ Nd/ ¹⁴⁴ Nd	0.5147 ± 30	0.1075 ± 64

Natural starting materials of pristine diabase (MF-R) and Archaean leucogneiss (FS-R) from east Greenland were ground with agate mortar under acetone for 20 min and dried at 120 °C under vacuum. Powders were doped with Sr and Nd by weighing appropriate amounts of reagent grade NdO and SrCO₃ (Aldrich Chemical Co.) and 92.1% ⁸⁷Sr SrCO₃ (batch 136990) and 97.51% ¹⁴⁴Nd NdO (batch 179601) obtained from Oak Ridge National Laboratories and fused. Mixes were ground for an additional 20 min and stored with a desiccant. Analytical uncertainties represent 2σ precision error limits for measured isotope ratios by ion microprobe. Calibration and operating procedures for the Cameca IMS 3f ion microprobe used here are discussed by Shimizu and Hart²¹ and Leshner¹⁹.

An attempt to address this question is shown in Fig. 2, which shows chemical and isotopic relationships between hybrid mafic (A) and felsic (B) compositions related by interdiffusion, using the results of Fig. 1 as a guide, and assuming that the magmas remain wholly liquid. At the initial stage (*t*₀) magmas A and B are brought into physical contact, providing the opportunity for diffusional exchange. The solid curves in Fig. 2*a, b* show the evolution of mafic hybrids (upper trajectories) and complementary felsic hybrids (lower trajectories) as interdiffusion acts to reduce compositional differences. Figure 2*a* indicates an early stage (*t*₀ → *t*₁) of 'uphill' diffusion of Sr into the mafic reservoir. For mobile components, such as Sr, this stage of selective exchange continues until activity differences are minimized between the chemical reservoirs^{2,3,15}. Limitations on partitioning of mobile elements between magmas of contrasting composition are provided by silicate liquid immiscibility^{17,18}, isothermal interdiffusion³ and Soret diffusion¹⁹ studies. Subsequent changes in composition become rate-limited by interdiffusion of network-former species; thus from stages *t*₁ to *t*₃ the Sr concentration is inversely correlated with silica content. The final (equilibrium) state, when the mixture of A and B is fully blended to form a single liquid of intermediate composition AB, is marked by *t*₃. The dashed line drawn between end-member compositions A and B represents the full spectrum of magma compositions that can be produced by simple binary mixing¹. Only if the activities of Sr in magmas A and B were, fortuitously, initially equal, would the irreversible mixing paths for complementary mafic and felsic hybrids closely follow the bulk-mixing curve. Concomitant crystallization involving, for example, plagioclase, in which Sr is a compatible component, will tend to displace the hybridization paths depicted in Fig. 2*a* further from the bulk-mixing hyperbola. However, unless the crystallizing phase is physically removed from the system, both the felsic and mafic hybridization paths must eventually converge to a single composition lying on the bulk-mixing hyperbola.

Figure 2*b* shows covariations of Sr and Nd isotope ratios with time for the same system. Note that the mafic and felsic hybrids initially evolve along shallow trajectories reflecting more rapid equilibration of Sr isotopes compared to those of Nd, as shown by the present experiments. Again, material balance requires that complementary hybridization paths converge to a

single composition on the bulk-mixing hyperbola dictated by the initial volume proportions of unmodified end-members. Figure 2*b* also shows that the time (*t*) required for Sr and Nd isotopic equilibrium to be achieved is less than that required for the admixture to homogenize completely (namely, *t*₃), which is again consistent with the results of Fig. 1.

The solid and dashed curves in Fig. 2 represent very different aspects of the mixing process; the bulk-mixing relations depict the compositional diversity of mixtures once equilibrium is attained for all volume proportions of the end-members, whereas the irreversible mixing paths define routes that components of a given mix take to reach a homogenized state. For example, using the diffusivities reported in Fig. 1 and the equation for diffusion from a stirred solution of limited volume presented by Crank²⁰, one-metre-sized blobs of felsic magma B entrained in molten basalt A would require a few thousand years to achieve Sr and Nd isotopic equilibrium with host A, although more than ten thousand years would be required to assimilate the blobs completely. As the homogenization time is proportional to the square of the diffusive mixing distance, increasing the size of blobs by one order of magnitude increases homogenization times by two orders of magnitude, further reducing the likelihood of complete homogenization on convective timescales and thus favouring selective contamination effects.

Viewing mixing and assimilation in this manner brings out some of the difficulties petrologists face in proving or disproving crustal effects in magmas, and in uniquely identifying end-members when bulk mixing is assumed. More sophisticated models coupling assimilation with crystallization (AFC) have been devised, but even these generally require that the contaminant be ingested in bulk. One needs to bear in mind, however, that the efficiency of mixing depends on magma kinetics and rheology, and that bulk-mixing relations accurately reflect only the end stage of a protracted history. Here I emphasize the importance of considering kinetic factors in modelling magma evolution and provide examples of decoupling of chemical and isotopic exchange when the mixing of mafic and felsic magmas is rate-limited by diffusion. These considerations may help to explain some of the discrepancies between geochemical data and predictions based on bulk-mixing (or AFC) models. Recognizing that hybrids are the product of selective exchange will offer additional constraints on the range of possible end-member compositions from which they evolved, and may provide information regarding the length and timescales on which mixing and contamination of magmas occur. □

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- Langmuir, C. H., Vocke, R. D. Jr, Hanson, G. N. & Hart, S. R. *Earth planet. Sci. Lett.* **37**, 380–392 (1978).
- Rosing, M., Leshner, C. E. & Bird, D. *Geology* **17**, 626–629 (1989).
- Watson, E. B. *Contr. Miner. Petrol.* **80**, 73–87 (1982).
- Johnston, A. D. & Wyllie, P. J. *Contr. Miner. Petrol.* **98**, 352–362 (1988).
- Zhang, Y., Walker, D. & Leshner, C. E. *Contr. Miner. Petrol.* **102**, 492–513 (1989).
- Leshner, C. E. & Walker, D. *Geochim. cosmochim. Acta* **50**, 1397–1411 (1986).
- Baker, D. R. *Geochim. cosmochim. Acta* **53**, 3015–3023 (1989).
- Leshner, C. E. & Walker, D. *Diffusion, Atomic Ordering, and Mass Transport: Selected Problems in Geochemistry* (ed. Ganguly, J.) (Springer, New York, in the press).
- Hofmann, A. W. in *Physics of Magmatic Processes* (ed. Hargraves, R. B.) 385–418 (Princeton University Press, 1980).
- Oldenburg, C. M., Spera, F. J., Yuen, D. A. & Sewell, G. J. *geophys. Res.* **94**, 9215–9236 (1989).
- Huppert, H. E. & Sparks, R. S. J. *Contr. Miner. Petrol.* **75**, 279–289 (1980).
- McBirney, A. R., Baker, B. H. & Nilson, R. H. *J. Volc. geotherm. Res.* **24**, 1–24 (1985).
- Sparks, R. S. J. & Marshall, L. A. *J. Volc. geotherm. Res.* **29**, 99–124 (1986).
- Bacon, C. R. & Metz, J. *Contr. Miner. Petrol.* **85**, 346–365 (1984).
- Grove, T. L., Kinzler, R. J., Baker, M. B., Donnelly-Nolan, J. M. & Leshner, C. E. *Contr. Miner. Petrol.* **99**, 320–343 (1988).
- Wiebe, R. A. *J. Petrol.* **29**, 383–411 (1988).
- Watson, E. B. *Contr. Miner. Petrol.* **56**, 119–134 (1976).
- Ryerson, F. J. & Hess, P. C. *Geochim. cosmochim. Acta* **42**, 921–932 (1978).
- Leshner, C. E. *J. geophys. Res.* **91**, 6123–6141 (1986).
- Crank, J. *The Mathematics of Diffusion* (Clarendon, Oxford, 1975).
- Shimizu, N. & Hart, S. R. *Rev. Earth planet. Sci.* **10**, 483–526 (1982).
- Shewmon, P. G. *Diffusion in Solids* (McGraw-Hill, New York, 1963).
- Leshner, C. E. *Eos* **69**, 1509 (1988).

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Influence of seasonal migration on geographic distribution of mitochondrial DNA haplotypes in humpback whales

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HUMPBAC whales (*Megaptera novaeangliae*) migrate nearly 10,000 km each year between summer feeding grounds in temperate or near-polar waters and winter breeding grounds in shallow tropical waters¹. Observations of marked individuals suggest that major oceanic populations of humpback whales are divided into a number of distinct seasonal subpopulations which are not separated by obvious geographic barriers^{2,3}. To test whether these observed patterns of distribution and migration are reflected in the genetic structure of populations, we looked for variation in the mitochondrial DNA of 84 individual humpback whales on different feeding and wintering grounds of the North Pacific and western North Atlantic oceans. On the basis of restriction-fragment analysis, we now report a marked segregation of mitochondrial DNA haplotypes among subpopulations as well as between the two oceans. We interpret this segregation to be the consequence of maternally directed fidelity to migratory destinations.

Using a small biopsy dart⁴, skin samples were collected from free-ranging humpback whales on two feeding grounds, southeastern Alaska ($n=20$) and central California ($n=20$), and on a single wintering ground, Hawaii ($n=16$) in the North Pacific, and on a single feeding ground, the Gulf of Maine ($n=28$) in the North Atlantic (Fig. 1). Total cellular DNA was

TABLE 1 Nucleotide divergence (%) of mtDNA within and between subpopulations of humpback whales

	SEA	HI	CCA	GOM
Southeastern Alaska (SEA)	0.000	0.000	0.176	0.282
Hawaii (HI)	0.014	0.028	0.156	0.262
Central California (CCA)	0.274	0.267	0.195	0.112
Gulf of Main (GOM)	0.380	0.374	0.308	0.196

Diagonal shows nucleotide diversity within a geographic region. Values below the diagonal are genetic distances between subpopulations uncorrected for within-region divergence. Values above the diagonal show genetic distances between regions after correcting for within-region divergence. All values were derived using the computer program MAXLIKE, courtesy of M. Nei and L. Jin, Center for Demographic and Population Genetics, University of Texas.

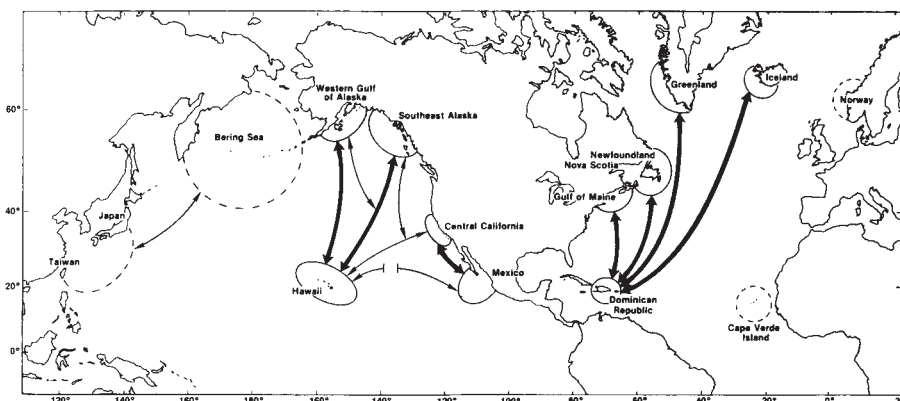
extracted from the skin samples and used for restriction-fragment analysis following Southern blotting and hybridization with a radioactively labelled molecular clone of porpoise mitochondrial (mt) DNA (Fig. 2). We scored a total of 81 to 85 restriction fragments per individual, surveying an average of 468 nucleotides, or 2.84% of the ~16,500 base pairs in the humpback whale mtDNA. The simple structure of the restriction-fragment length patterns allowed us to infer the presence of 88 independent restriction sites, of which 9 were polymorphic. The sex of each individual was identified by Southern hybridization of *Eco*RI-restricted total cellular DNA with the clone pDP1007, which is derived from the human Y chromosome⁵.

Among the 84 individuals surveyed, we found 12 mtDNA haplotypes that differed from one another by between 1 and 7 restriction sites. The average nucleotide divergence (that is, π , nucleotide diversity⁶) among all individuals was 0.248% (s.d. = 0.0151%) and ranged from 0.00% to 0.196% within each geographic subpopulation (Table 1). Genetic distance (d , the divergence between subpopulations after adjustment for within-subpopulation diversity⁶) between subpopulations indicated that southeastern Alaska and Hawaii are essentially identical to one another and diverged from the Gulf of Maine by 0.26–0.28%; California is intermediate between the Gulf of Maine and Hawaii/southeastern Alaska, diverging from each by 0.11–0.18%. There were no significant biases in the geographic distribution or haplotype frequencies of the sexes (C.S.B. *et al.*, manuscript submitted).

A topological network of restriction-site changes generated by maximum parsimony showed a striking agreement between the geographic distribution of haplotypes and previously reported patterns of migratory movement (Fig. 3). There was no overlap in haplotypes from the two oceanic populations, or

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FIG. 1 The migratory destinations and population structure of humpback whales in the North Pacific and western North Atlantic oceans, based on observations of marked individuals^{2,3,7,23–26}. Regions encircled by a solid line are defined by current observations of seasonal return by naturally marked individuals. Regions encircled by a broken line are defined by historical patterns of distribution during periods of commercial whaling. Arrows connect seasonal habitats visited by individually identified whales but do not necessarily indicate migratory routes. Thick arrows connect regions with known strong migratory interchange and thin arrows connect regions with weak migratory interchange. The broken line connecting Hawaii and Mexico indicates the probable presence of an intervening seasonal migration to a feeding ground by individuals sighted on both winter grounds in alternate years.



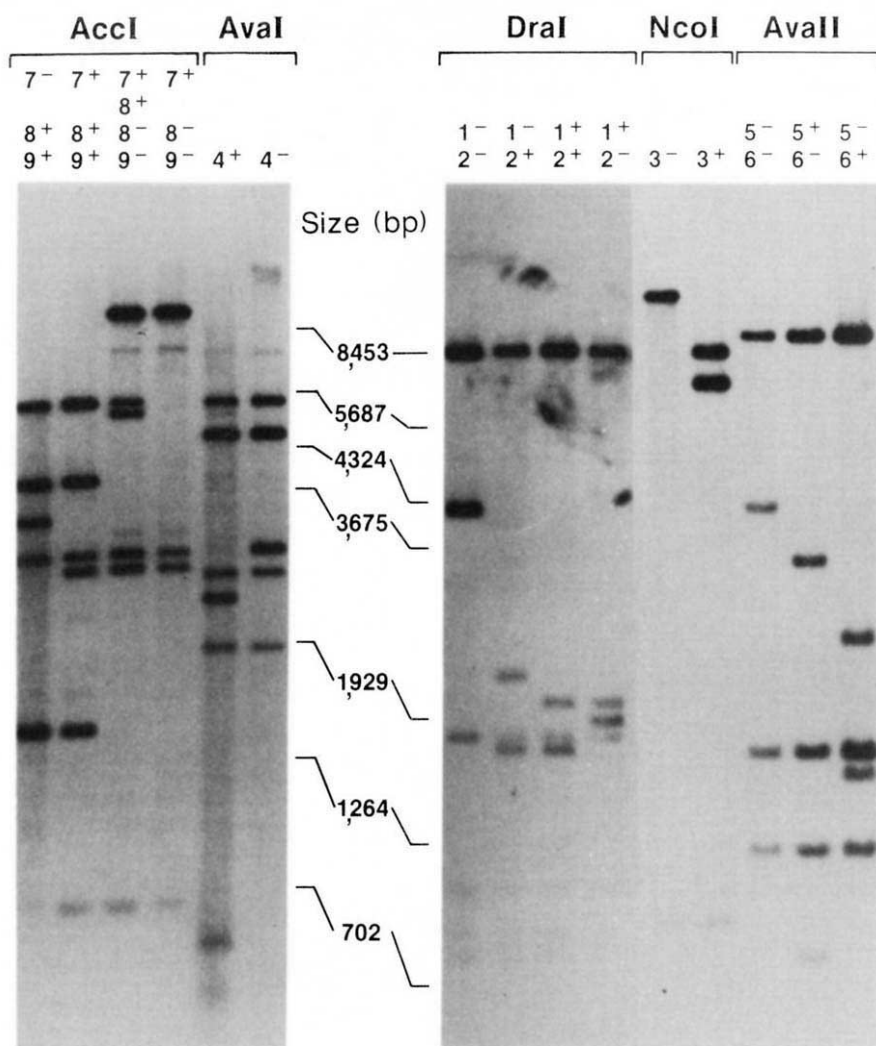


FIG. 2 Polymorphic restriction-fragment sites defining the 12 mtDNA haplotypes of humpback whales. The presence (+) or absence (-) of the 9 restriction sites are noted for each of the 15 unique restriction-fragment patterns. A 0.2-kb fragment in the 1⁺, 2⁺ pattern of *DraI* is visible on longer autoradiographic exposures. A single individual from Hawaii, referred to as haplotype B in Fig. 3, appeared to be heteroplasmic for restriction site 8 (indicated by 8⁺, 8⁻)²⁷. Migration of calibration fragments is indicated.

METHODS. About 1.5 µg of total cellular DNA from each individual was digested with 18 informative restriction enzymes (*AccI*, *AvaI*, *Avall*, *BamHI*, *BclI*, *BglI*, *BglII*, *BstEII*, *DraI*, *HaeIII*, *HincII*, *HindIII*, *HpaI*, *MspI*, *NcoI*, *SstI*, *SstII* and *XbaI*). Restricted DNA was separated on the basis of molecular weight by electrophoresis in 0.8% or 1% agarose gels and transferred to nylon filters by Southern blotting in 10 × SSC. Filters were hybridized at 65 °C in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA, with radioactively labelled mtDNA from a Dall's porpoise, *Phocoenoides dalli* (gift from E. Bermingham and W. O. McMillan) that was cloned into a λ-phage vector. Filters were washed at a final stringency of 0.1 × SSC, 0.5% SDS at 50 °C for 30 min and autoradiographed for 1–3 days at -70 °C.

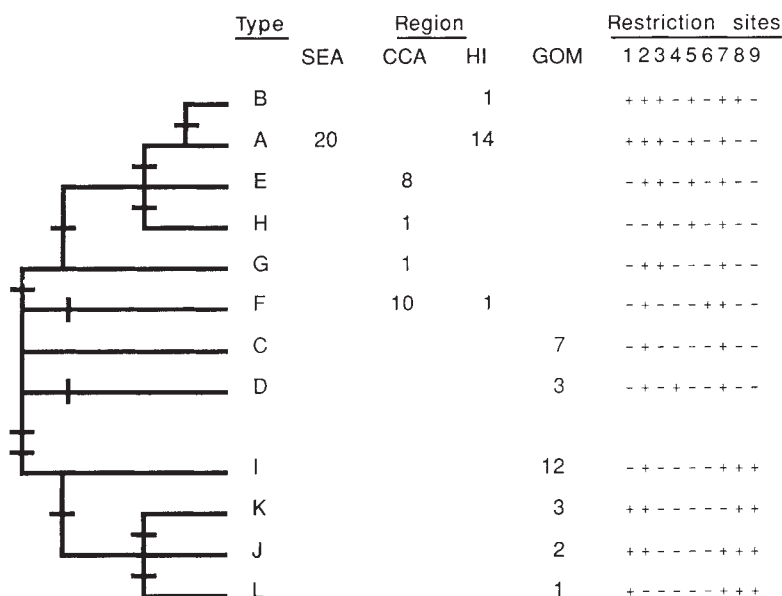


FIG. 3 Topological network of humpback whale mtDNA haplotypes from southeastern Alaska (SEA), central California (CCA), Hawaii (HI) and the Gulf of Maine (GOM). The frequency of haplotypes in each geographic region and the presence (+) or absence (-) of polymorphic restriction sites defining each haplotype are shown adjacent to the branch termini. Polymorphic restriction sites are numbered according to Fig. 2. Restriction site changes are indicated by vertical or horizontal bars along the network branches. This network is in close agreement with the consensus tree of 50 equally parsimonious trees (consistency index of 0.75) generated by the Phylogenetic Analysis using Parsimony computer program (courtesy of D. Swofford, Illinois Natural History Survey, Champaign, Illinois).

between the southeastern Alaska and California feeding grounds in the North Pacific. The Hawaiian wintering ground was dominated by the southeastern Alaska haplotype A, but also contained one individual with an F haplotype, the most common type in the central California sample. This supports earlier observations that individuals from different feeding grounds

congregate on a common wintering ground^{2,3,7,8}. A computer simulation indicated that such a strong geographic segregation of haplotypes would be expected by chance less than once in 500 samples from a single panmictic population⁹.

Given the tremendous mobility of humpback whales and the apparent absence of geographic barriers within oceans, we sug-

gest that the complete segregation of mtDNA types on the North Pacific feeding grounds reflects the result of strong maternal traditions in migratory destinations^{10,11}, perhaps reinforced by the social or genetic advantages of philopatry¹²⁻¹⁴. These results emphasize the importance of considering behavioural strategies such as migration and natal fidelity¹⁵⁻¹⁷, as well as dispersal¹⁸, in describing the population structure of marine species.

Despite the marked segregation of mtDNA haplotypes in populations of humpback whales, average nucleotide diversity was low compared with most other mammals similarly studied¹⁹, and genetic distance between North Pacific and Atlantic populations was small, considering the 3-Myr separation of these oceans by the Isthmus of Panama²⁰ and assuming a 1-2% Myr⁻¹ rate of mtDNA divergence^{19,21}. This pattern of low but highly

structured variation could be explained by a deceleration of mtDNA evolution in humpback whales, or by periodic gene flow between oceans followed by a rapid assortment of maternal lineages within oceans. A possible avenue for gene flow between oceanic populations is indicated from the logbooks of nineteenth century whaling ships, which report that northern and southern hemisphere populations overlap along the equatorial Pacific coasts of South America²². Further analysis of mtDNA from individuals in subpopulations of the southern hemisphere, as well as analysis of nuclear gene markers from various populations, could provide a global description of gene flow in the present populations of humpback whales and allow a reconstruction of the historic processes that preceded the genetic subdivisions of modern populations. □

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1. Mackintosh, N. A. *The Stocks of Whales* (Fishing News (Books), London, 1965).
2. Katona, S. K. & Beard, J. A. *Rep. Int. Whal. Commn* (Special Issue) **12** (in the press).
3. Baker, C. S. *et al. Mar. Ecol. Prog. Series* **31**, 105-119 (1986).
4. Lambertsen, R. H. *J. Mamm.* **68**, 443-445 (1987).
5. Page, D. C. *et al. Cell* **51**, 1091-1104 (1987).
6. Nei, M. *Molecular Evolutionary Genetics* (Columbia University Press, New York, 1987).
7. Darling, J. D. & McSweeney, D. J. *Can. J. Zool.* **63**, 308-314 (1985).
8. Mattila, D. K. *et al. Can. J. Zool.* **67**, 281-285 (1989).
9. Palumbi, S. R. & Wilson, A. C. *Evolution* (in the press).
10. Baker, C. S., Perry, A. & Herman, L. M. *Mar. Ecol. Prog. Series* **41**, 103-114 (1987).
11. Clapham, P. J. & Mayo, C. A. *Can. J. Zool.* **65**, 2853-2863 (1987).
12. Baker, C. S. thesis, Univ. Hawaii (1985).
13. Hamilton, W. D. *J. theor. Biol.* **7**, 1-52 (1964).
14. Waser, P. M. & Jones, W. T. *Q. Rev. Biol.* **58**, 355-390 (1983).
15. Bowen, B. W., Meylan, A. B. & Avise, J. C. *Proc. natn. Acad. Sci. U.S.A.* **86**, 573-576 (1989).
16. Saunders, N. C., Kessler, L. G. & Avise, J. C. *Genetics* **112**, 613-627 (1986).
17. Avise, J. C., Helfman, G. S., Saunders, N. C. & Hales, L. S. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4350-4354 (1986).
18. Avise, J. C. *et al. A. Rev. Ecol. Syst.* **18**, 489-522 (1987).

19. Wilson, A. C. *et al. Biol. J. Linn. Soc. Lond.* **26**, 375-400 (1985).
20. Savage, D. E. *Mammalian Paleofaunas of the World* (Addison-Wesley, London, 1983).
21. Brown, W. M. *Evolution of Genes and Proteins* (eds Nei, M. & Koehn, R. K.) 62-88 (Sinauer, Sunderland, Massachusetts, 1983).
22. Townsend, C. H. *Zoologica* **19**, 1-50 (1935).
23. Whitehead, H. *Rep. Int. Whaling Commn* **32**, 345-353 (1982).
24. Martin, A. R. *et al. J. Mamm.* **65**, 330-333 (1984).
25. Perry, A., Mobley, J. R., Baker, C. S. & Herman, L. M. *Sea Grant Miscellaneous Report UNH-SEAGRANT-MR-88-02* (Office of Sea Grant, Honolulu, 1988).
26. Nishiwaki, M. *Sci. Rep. Whales Res. Inst. Tokyo* **14**, 49-86 (1959).
27. Laipis, P. J., Van de Walle, M. J. & Hauswirth, W. E. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8107-8110 (1988).

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Muscarinic modulation of a transient K⁺ conductance in rat neostriatal neurons

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NEURONS of the neostriatum are richly innervated by cholinergic neurons of intrinsic origin¹⁻³. Both pre- and post-synaptic muscarinic receptors mediate the effects of acetylcholine (ACh)⁴⁻⁶. Activation of these receptors is functionally significant, particularly in Parkinson's disease⁷. Current-clamp studies indicate that muscarinic receptors serve to decrease the responsiveness of neostriatal neurons to excitatory inputs⁸⁻¹⁰. Here we present evidence that this effect is caused, in part, by the muscarinic modulation of the A-current, a transient outward potassium current. The voltage dependence of this current suggests that normally it enhances spike repolarization and slows discharge rate, but does not affect 'synaptic integration'. We find that under the influence of muscarinic agonists, the voltage dependence of A-current activation and inactivation is shifted towards more negative membrane potentials and the peak conductance is increased. Therefore, at relatively hyperpolarized resting potentials, ACh transiently alters the functional role of the A-current, allowing it to suppress excitatory inputs and further slow the discharge rate. But at relatively depolarized resting potentials, ACh increases excitability by removing the A-current through inactivation.

We studied the effects of muscarinic agonists on the A-current in cultured rat neostriatal neurons using whole-cell voltage-clamp techniques¹¹. We maintained neurons from either late embryonic (E17) or postnatal (P1-7) rat pups *in vitro* for 3-21 days¹². Previous electrophysiological studies of these

neurons^{13,14} have shown that in the presence of Na⁺ and Ca²⁺ channel blockers, the outward K⁺ currents activated by depolarization are primarily due to a sustained tetraethylammonium (TEA)-sensitive current and a transient 4-aminopyridine (4-AP)-sensitive current. The transient current has a pharmacology and ionic selectivity similar to those of the A-current in other mammalian brain neurons, but differs in voltage dependence¹⁴. In most neurons dialysed with 150 mM internal potassium, the half-activation and half-inactivation voltages of the A-current are 20-30 mV more positive than those of hippocampal neurons^{15,16} and resemble those of the A-current in mammalian heart cells^{17,18} or *Drosophila*^{19,20}.

The application of the stable nonhydrolyzable cholinergic agonist, carbachol, produced a reversible increase in the amplitude of the transient component of the outward K⁺ current (Fig. 1a, left). Inactivation of the A-current with a prepulse to -40 mV revealed that the sustained current was unaffected (Fig. 1a, right). The response seemed to be relatively slow in onset and offset (Fig. 1, inset). A similar result was obtained in 73% (11 out of 15) of the neurons studied in the absence of external TEA. In 20% (3 out of 15) of this sample, carbachol also increased the amplitude of the sustained outward current. The holding current at -70 mV was not affected. The increase in the transient current induced by carbachol was unaltered by bath application of TEA (20 mM; *n* = 20), but was blocked by 4-AP (5 mM; *n* = 3).

Although carbachol is a mixed cholinergic agonist, its actions were mediated by muscarinic receptors. The enhancement of A-current by carbachol was unaffected by inclusion of the nicotinic antagonist mecamylamine (10 μM) in the bath. By contrast, the muscarinic antagonist atropine (10 μM; *n* = 3) blocked the carbachol response (Fig. 1b). We also observed the effect of carbachol on the A-current with the specific muscarinic agonist, muscarine (Fig. 2 and Table 1). Although selective antagonists were not tested, binding studies on intact neostriatal cultures have shown that muscarinic receptor expression is limited to primarily the M1 subtype²¹.

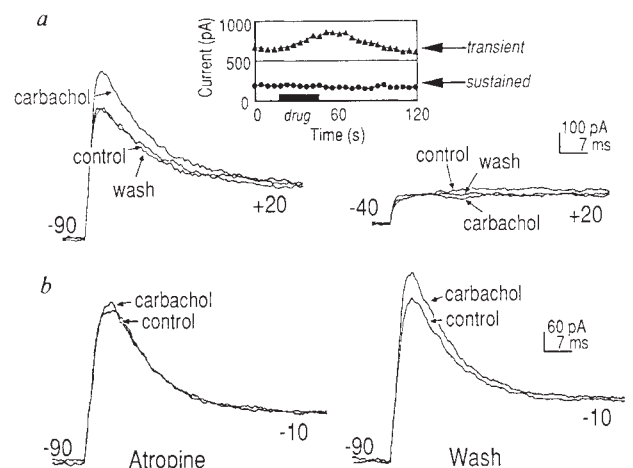
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TABLE 1 Effects of muscarine treatment on A-current voltage dependence

	$V_{h_{act}}$ (mV)	$V_{c_{act}}$ (mV)	$V_{h_{inact}}$ (mV)	$V_{c_{inact}}$ (mV)	τ_{inact} (ms)
Control	1.9 ± 1.0	14.0 ± 0.4	-50.6 ± 0.6	5.1 ± 0.5	18.0 ± 2.1
Muscarine	-12.3 ± 2.3	14.5 ± 0.8	-63.8 ± 2.4	5.4 ± 0.2	19.5 ± 2.6

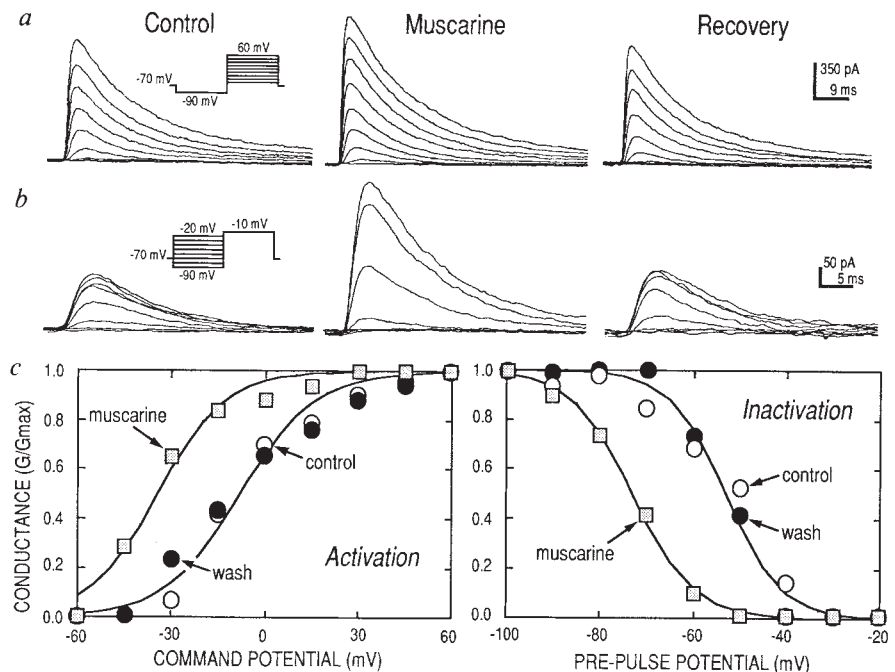
Ten neurons were analysed. Both activation (act) and inactivation (inact) data were obtained from seven cells; from three other cells, additional data were obtained. Activation ($n=10$) and inactivation curves ($n=7$) were constructed from peak currents before and after 1-min application of muscarine ($50 \mu\text{M}$). The curves were fitted with Boltzmann functions (see Fig. 2c). Using extrapolated ($t=0$) current amplitudes instead of peak currents, both control and drug curves were shifted ~ 10 mV to the left; this similarity was a reflection of the small differences in activation rates in these two conditions (see below). Inactivation time constants (τ_{inact}) were calculated from mono-exponential least-squares fits to the decay phase of currents evoked by a command to $+60$ mV. Fits to the decay phase of currents evoked near the half-activation potential before and after drug application yielded qualitatively similar results. V_c , Boltzmann slope factor; $V_{h_{act}}$ and $V_{h_{inact}}$, half-activation and half-inactivation voltages, respectively.

FIG. 1 Muscarinic enhancement of the A-current. *a*, Carbachol ($100 \mu\text{M}$) increased the transient outward current (left) evoked by a step to $+20$ mV after a pre-pulse to -90 mV (500 ms). Washout of carbachol returned current amplitudes to control levels. The sustained current, isolated with a conditioning pulse to -40 mV (right), was unaffected. Inset shows that carbachol was applied for 30 s with a puffer pipette positioned $\sim 200 \mu\text{m}$ from the cell. The peak response occurred 10 s after drug application and recovered slowly during the wash period. TEA was omitted from the external solution in this experiment. *b*, In the presence of atropine ($10 \mu\text{M}$), carbachol ($100 \mu\text{M}$) produced only a small change in the transient current amplitude (left) evoked by stepping to -10 mV (pre-pulse to -90 mV). After 2 minutes of wash, carbachol application evoked a much larger increase in A-current (right). Leak and capacitive currents were not subtracted in *a* and *b*. METHODS. Cultures of embryonic rat neostriatum were prepared in a manner similar to that described previously¹². Cells from postnatal rat pups (1–7 days) were obtained using enzymatic digestion (pronase; 3 mg ml^{-1}) with a protocol similar to that of Mody *et al.*²⁹. Whole-cell recordings were performed as described previously^{13,14}. The external recording solution contained (in mM): NaCl, 96; TEA-Cl, 20; KCl, 10; Na-HEPES, 10; MgCl_2 , 1; CaCl_2 , 2; tetrodotoxin, 1×10^{-3} ; CdCl_2 , 0.2; glucose, 36. The pH was adjusted to 7.4 with HCl, and the osmolarity adjusted to 300 mosmol. Mecamylamine ($10 \mu\text{M}$) was included in all carbachol experiments to block nicotinic depolarization. In initial experiments, NaCl replaced TEA-Cl on an equimolar basis. The internal recording solution contained (in mM): KCl, 125; MgCl_2 , 2; HEPES buffer, 10; EGTA, 5; $\text{Na}_2\text{-ATP}$, 2; $\text{Na}_3\text{-GTP}$, 0.2. The pH was adjusted to 7.3 with KOH, and the osmolarity adjusted to 260–270 mosmol. Agonists



were applied by pressure ejection from drug electrodes ($5\text{--}8 \mu\text{m}$ tip diameter) positioned $\sim 200 \mu\text{m}$ from the neuron; during drug application, a background flow of control solution was maintained to minimize washout times. The records in *a* were taken from a neuron cultured from a P7 rat maintained *in vitro* for 6 days; those in *b* were taken from an E17 cell maintained *in vitro* for 20 days.

FIG. 2 Muscarinic agonists produced a negative shift in the voltage dependence of A-current gating. *a*, At the left, control currents were evoked by depolarizing command steps (inset) after a pre-pulse to -90 mV (500 ms). Application of muscarine ($50 \mu\text{M}$) (centre), increased current amplitudes evoked by the same protocol, particularly at relatively negative potentials. After washing, current amplitudes returned to control levels (right). *b*, The steady-state inactivation of the A-current was studied with a standard pre-pulse protocol (-100 to -20 mV, 500 ms) with a test step to -10 mV. Under control conditions (left), the A-current activated at -10 mV showed little inactivation with pre-pulse potentials more negative than -60 mV (top three traces). Considerable inactivation occurred in this range after muscarine treatment (middle). The voltage dependence of inactivation recovered after drug washout (right). *c*, Steady-state activation and inactivation curves constructed from the data in *a* and *b* illustrate the negative shift in voltage dependence produced by muscarine. Steady-state amplitudes were determined by extrapolation back to command onset from the exponential decay phases of the currents after subtraction of the current component not inactivated by holding at -40 mV. Conductances were computed and normalized on the assumption that currents were carried by potassium¹⁴. Data points were fitted with a Boltzmann function: for activation, $G=1/(1+\exp(-(V-V_h)/V_c))$, and for inactivation, $G=1/(1+\exp((V-V_h)/V_c))$, where G is the normalized conductance, V is the command potential for activation or the conditioning potential for inactivation and V_c is the Boltzmann slope factor. The half-



activation and half-inactivation voltages (V_h) under control conditions were -8 mV and -53 mV, respectively. Muscarine treatment shifted the V_h to -34 mV and -73 mV, respectively. Data were from an embryonic neuron cultured for 18 days. Series resistance compensation, $\sim 70\%$; capacitive and leak currents were subtracted.

The muscarinic enhancement shown in Fig. 1 could have been mediated by a negative shift in voltage dependence, an increase in maximum conductance, or a slowing of inactivation rates²². No consistent change in A-current inactivation time constant was observed after carbachol application (Table 1). Because of space-clamp limitations in older cells, this result must be interpreted with caution. In 50% (5 out of 10) of responsive neurons, a small increase (~25%) was seen in the peak conductance. The most consistent effect, seen in all responsive neurons (10 out of 10), was a negative shift in the voltage dependence of activation and inactivation.

The characteristics of this shift are shown in Fig. 2. We activated the A-current with depolarizing voltage pulses from a holding potential of -90 mV (Fig. 2a). During muscarine application, currents evoked by moderate depolarizations were particularly enhanced. Washing off the muscarine returned currents to control levels. In Fig. 2c (right), the normalized maximum conductance are plotted as a function of the command potential and fitted with a Boltzmann function. These plots show that the voltage dependence of activation was reversibly shifted towards more negative potentials by muscarine treatment without changing the steepness of the conductance-voltage relationship.

Figure 2b shows the changes in the voltage dependence of steady-state inactivation produced by muscarine. We inactivated the A-current by holding the cell at progressively more depolarized potentials, so less current was evoked during the test pulse (-10 mV). In the presence of muscarine, the A-current inactivated at more-hyperpolarized potentials than in control and wash conditions. In Fig. 2c (left), the normalized maximum conductances are plotted as a function of the pre-pulse potential and fitted with a Boltzmann function. As with activation, the voltage dependence of inactivation was reversibly shifted toward negative potentials without altering the steepness of the conductance-voltage relationship. In general, activation and inactivation voltage dependence was altered by 10–15 mV (Table 1). That the voltage dependencies of activation and inactivation were shifted towards the left and in parallel argues strongly that the drug effects did not arise from a space-clamp or series resistance artefacts. These shifts are similar to those reported by DiFrancesco and Tromba²³ for the inward rectifier in myocytes in response to ACh; they are, however, contrary to findings in hippocampal neurons²⁴.

The shift in the voltage dependence of steady-state inactivation indicates that if test pulses are delivered from depolarized holding potentials, then the A-current would seem to be reduced by ACh or muscarinic agonists, rather than increased. We found this to indeed be the case (Fig. 3): currents evoked by a command

to +20 mV were reduced by carbachol (100 μ M) when evoked from a -60 mV holding potential.

The characteristics of the muscarinic modulation of the A-current has several functional implications for neostriatal neurons. The principal neuron of the neostriatum, the medium spiny projection neuron, tends to reside in two different 'resting' membrane-potential ranges *in vivo*, depending on the status of cortical input; these ranges are centred around -75 mV and -55 mV (refs 25 and 26). At -75 mV, the cells are relatively unresponsive to synaptic inputs, and are quiescent; ACh, by shifting the activation range of the A-current into the subthreshold range, will stabilize this tendency^{27,28}. At these negative membrane potentials, muscarinic agonists would attenuate large excitatory post-synaptic potentials without altering the resting membrane potential or apparent electrotonic dimensions, as seen in current-clamp recordings from striatal slices^{8–10}. But, at -55 mV, where these neurons actively discharge, ACh, by shifting the inactivation voltage dependence of the A-current, will make the neuron more excitable by removing this outward current through inactivation. In both cases, ACh should stabilize the state of the neuron, not simply excite or inhibit it. □

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- Graybiel, A. M. & Ragsdale, C. W. Jr in *Chemical Neuroanatomy* (ed. Emson, P. C.) 427–504 (Raven, New York, 1978).
- Bolam, J. P., Wainer, B. H. & Smith, A. D. *Neuroscience* **84**, 711–718 (1984).
- DiFiglia, M. *J. comp. Neurol.* **255**, 245–258 (1987).
- Brann, M. R., Buckley, N. J. & Bonner, T. I. *FEBS Lett.* **230**, 90–94 (1988).
- Mash, D. C. & Potter, L. T. *Neuroscience* **19**, 551–564 (1986).
- Weiss, S. et al. *J. Neurochem.* **50**, 1425–1433 (1988).
- Calne, D. B. *Therapeutics in Neurology* (Blackwell, London, 1980).
- Akaike, A., Sasa, M. & Takaori, S. *J. Pharm. exp. Ther.* **246**, 1129–1136 (1988).
- Dodt, H. U. & Misgeld, U. *J. Physiol., Lond.* **380**, 593–608 (1986).
- Malenka, R. C. & Kocsis, J. D. *J. Neurosci.* **8**, 3750–3756 (1988).
- Hamill, O. P. et al. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Surmeier, D. J., Kita, H. & Kitai, S. T. *Dev. Brain Res.* **42**, 265–282 (1988).
- Surmeier, D. J., Bargas, J. & Kitai, S. T. *Brain Res.* **473**, 187–192 (1988).
- Surmeier, D. J., Bargas, J. & Kitai, S. T. *Neurosci. Lett.* **103**, 331–337 (1989).
- Numann, R. E., Wadman, W. J. & Wong, R. K. S. *J. Physiol., Lond.* **393**, 331–353 (1987).
- Segal, M., Rogawski, M. A. & Barker, J. L. *J. Neurosci.* **4**, 604–609 (1984).
- Giles, W. R. & Van Ginneken, A. C. G. *J. Physiol., Lond.* **368**, 243–264 (1985).
- Josephson, I. R., Sanchez-Chapula, J. & Brown, A. M. *Circulation Res.* **54**, 157–162 (1984).
- Salkoff, L. & Wyman, R. *Nature* **293**, 228–230 (1981).
- Solc, C. K. & Aldrich, R. W. *J. Neurosci.* **8**, 2556–2570 (1988).
- Akins, P. T., Surmeier, D. J. & Kitai, S. T. *J. Neurochem.* **54**, 266–273 (1990).
- Hille, B. *Ionic channels of excitable membranes* (Sinauer, Sunderland, Massachusetts, 1984).
- DiFrancesco, D. & Tromba, C. *J. Physiol., Lond.* **405**, 477–491 (1988).
- Nakajima, Y. et al. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3022–3026 (1986).
- Wilson, C. J. & Groves, P. M. *Brain Res.* **220**, 67–80 (1981).
- Wilson, C. J., Chang, H. T. & Kitai, S. T. *Exp. Brain Res.* **51**, 227–235 (1983).
- Byrne, J. H. *J. Neurophysiol.* **43**, 651–668 (1980).
- Cassell, J. F. & McLachlan, E. M. *J. Physiol., Lond.* **374**, 273–288 (1986).
- Mody, I., Salter, M. W. & MacDonald, J. F. *Neurosci. Lett.* **96**, 70–75 (1989).

Regulation and deregulation of cardiac Na^+ - Ca^{2+} exchange in giant excised sarcolemmal membrane patches

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A PLASMALEMMAL Na^+ - Ca^{2+} exchange mechanism^{1,2} is an important electrogenic determinant of contractility in cardiac cells^{3–5}. As in other cell types^{6–8}, calcium influx by Na^+ - Ca^{2+} exchange is secondarily activated by cytoplasmic calcium⁴ and probably ATP⁹, but these modulatory mechanisms are either absent or altered in isolated cardiac sarcolemmal vesicles^{5,12}. Involvement of a calcium-dependent protein kinase in exchange regulation has been suggested^{7,10} but not verified^{5,11}. Here I describe measurements of outward Na^+ - Ca^{2+} exchange current, corresponding to calcium influx, in giant excised sarcolemmal

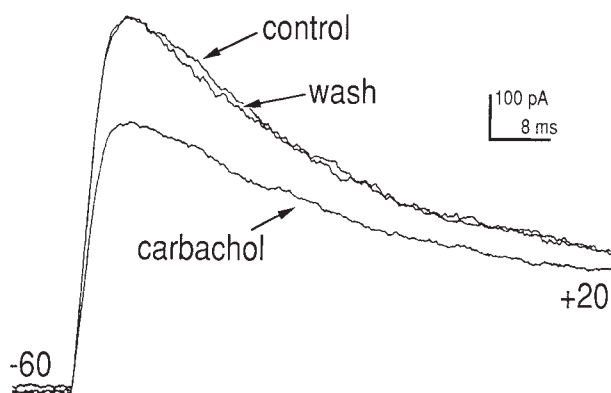


FIG. 3 The application of carbachol (100 μ M) leads to an attenuation of A-current amplitude when evoked from relatively depolarized potentials (-60 mV), as predicted from the change in steady-state inactivation. Depolarizing test pulse was to +20 mV. The neuron was cultured from embryonic neostriatum and maintained *in vitro* for 16 days. Recording methods as in Figs 1 and 2. Capacitative and leak currents were not subtracted.

patches¹¹ from guinea pig myocytes. The exchange current is stimulated by both calcium and Mg-ATP from the cytoplasmic face, evidently through separate mechanisms. Activation by cytoplasmic calcium takes place within seconds, is reversible, and does not require ATP. Stimulation by Mg-ATP reverses only slowly over >10 min, or not at all. Unexpectedly, a substantial decrease in exchange current occurs during activation by cytoplasmic sodium, which seems to reflect an inactivation process rather than ion concentration changes or a 'first pass' exchange cycle. This apparent inactivation, and the modulations by cytoplasmic calcium and Mg-ATP, are all abolished by brief treatment of the cytoplasmic surface with chymotrypsin, leaving the exchanger in a maintained state of high activity. Therefore, limited proteolysis deregulates Na^+ - Ca^{2+} exchange and could contribute to the loss of secondary regulation of the exchange in isolated sarcolemmal vesicles.

The conventional excised membrane patch method¹³ is not readily suited for the study of nonunitary currents such as those expected from ion pumps and exchangers, because the ratio of membrane surface area to nonspecific leak permeability is too small. Improvements of 1 to 2 log units are achieved by using cardiac cells pre-incubated in an EGTA/high KCl/dextrose solution to induce separation of sarcolemma from underlying structure¹¹. Seals of 5 to 20 G Ω are obtained with patches of 10 to 16- μm diameter, corresponding to 0.7–2 pF and 70–200 μm^2 . An outward Na^+ - Ca^{2+} exchange current was identified in excised inside-out patches¹¹ with the following characteristics, which are all consistent with exchange current measurements in whole myocytes. With sodium pump and channels blocked, the outward current is activated specifically by cytoplasmic sodium (that is in the bath solution) when calcium is included in the extracellular solution (that is, in the pipette). The current shows sigmoidal dependence on cytoplasmic sodium, is activated by cytoplasmic calcium, blocked by either cobalt or the organic Na^+ - Ca^{2+} exchange inhibitor, dichlorobenzamil, and has a high-temperature sensitivity ($Q_{10} > 2$).

Figure 1 illustrates the three types of secondary modulation of the exchange current that I observed in excised patches: apparent inactivation, long-term stimulation by Mg-ATP, and rapid activation by cytoplasmic calcium. Figure 1a shows the typical current transient obtained during a step increment of the cytoplasmic sodium concentration from 0 to 60 mM at 0 mV, replacing CsCl for NaCl. Conditions were chosen to minimize the effects of changes in ion concentrations, which could arise during exchange activity if diffusion restrictions exist on either side of the patch. Extracellular calcium was buffered at 7.5 mM with 55 mM malate–30 mM calcium. Cytoplasmic

free calcium was buffered at 0.2 μM with 30 mM BAPTA 30 mM bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid/20 mM calcium. The extracellular sodium concentration was 60 mM, so there was no sodium gradient on application of 60 mM cytoplasmic sodium. Also, 10 mM KCl was included in solutions on both sides, because in different cell types potassium can modulate¹⁴ and/or participate in¹⁵ Na^+ - Ca^{2+} exchange. Therefore, at the 0 mV holding potential, the calcium gradient alone was available to drive the exchange current under these conditions. A peak current of 42 pA was reached on application of sodium, and the current declined by ~80%, with a time constant of ~1 s. This behaviour was not modified by varying the degree of calcium buffering on either side (for example, with no extracellular calcium buffer and with cytoplasmic EGTA varying from 0.2 to 30 mM), by removing extracellular sodium (all further records), or by using lithium instead of caesium as a sodium substitute (for example, Fig. 1b). (A decrease in exchange current during application of cytoplasmic sodium was not described in the initial report¹¹, because methodological causes of current transients had not then been eliminated. In those experiments, a transient current component was probably 'blunted' by several factors, including relatively slow solution changes and the averaging of data over several seconds. Also, it is now known that contamination of 'sodium-free' superfusion solutions by 3 to 6 mM sodium can markedly attenuate the transient current component.) It is important to note that current decay ($t_{1/2} \geq 1\text{ s}$) is far too slow for a first pass exchange cycle¹². Exchanger densities $>1 \times 10^6$ per μm^2 would be needed to account for the currents recorded.

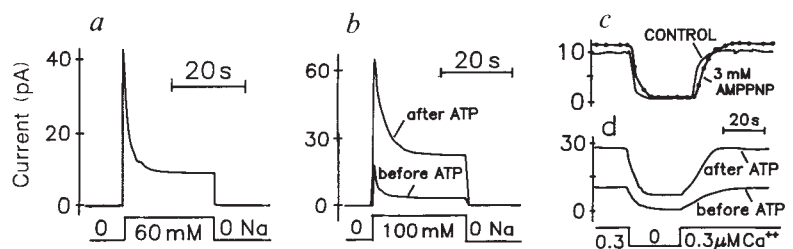
Figure 1b demonstrates long-term stimulation of exchange current transients by cytoplasmic Mg-ATP, typical for more than 20 patches taken from cells that had shortened in the Ca^{2+} -free, EGTA/KCl/dextrose solution. These cells were very probably depleted in ATP. Brief application of 1 to 10 mM Mg-ATP in such patches increased the exchange current several fold with little or no reversal of the effect during the lifetime of the patch (15–40 min). The control transient shown in Fig. 1b ('before ATP') was the last of several stable records with 80% transient current. The larger current record ('after ATP') was obtained 2 min after 5 mM Mg-ATP had been superfused for 1 min.

At 37 °C, steady-state outward current was turned off by removing cytoplasmic calcium ($t_{1/2} = 0.6\text{--}3\text{ s}$) and turned on by reapplication of calcium ($t_{1/2} = 2\text{--}6\text{ s}$) (Fig. 1c, d). Because activation by calcium could reflect a calcium-dependent phosphorylation^{7,10}, I tested for the existence of residual ATP in the patches by performing the experiments described in Fig. 1c entirely in

FIG. 1 Secondary modulation of outward Na^+ - Ca^{2+} exchange current in giant excised inside-out patches (37 °C, 0 mV). **a**, Current transient activated with 60 mM Na^+ (substituted for 60 mM Cs^+). Cytoplasmic Ca^{2+} , 0.2 μM with 30 mM BAPTA/20 mM CaCl_2 ; extracellular Ca^{2+} , 7.5 mM with 50 mM malate/30 mM CaCl_2 ; 60 mM extracellular NaCl; 10 mM KCl on both sides. **b**, Current transients before and after application of 5 mM Mg-ATP for 1 min (100 mM NaCl substituted for LiCl). Cytoplasmic Ca^{2+} , 0.3 μM . **c** and **d**, Time courses of current inhibition by removal and re-addition of 0.3 μM cytoplasmic Ca^{2+} . Cytoplasmic Na^+ , 100 mM. **c**, As indicated, with and without 3 mM AMPPNP. **d**, As indicated, before and after application of 5 mM Mg-ATP for 1 min.

METHODS. Two modifications from a previous report¹¹. Pipette tips (thin-walled borosilicate glass) were coated by passing them through a thin film of a Parafilm/mineral oil mixture drawn into air on a glass rod. Solution changes here and in Fig. 2 were made using a double-barrelled theta-style pipette to continuously superfuse the patch electrode tip with either of two solutions. Peak exchange currents were usually achieved within <100 ms during activation by cytoplasmic sodium. All solutions contained 25 mM CsCl and 25 mM tetraethylammonium chloride to block potassium channels; extracellular (pipette) solutions contained 1 mM 4-aminopyridine, 1 mM BaCl_2 , 10 μM verapamil and 25 μM ouabain to block channel mechanisms and the sodium pump. Except for **a**, cytoplasmic solutions (that is, those

solutions superfusing the cytoplasmic membrane face) also contained 25 mM HEPES buffer, 22 mM EGTA, CaCO_3 or CaCl_2 from calibrated stock solutions to achieve the desired free calcium concentrations (discrepancies were $\leq 15\%$ between calculated values $<2\text{ }\mu\text{M}$ and values measured with fura-2), 1 mM MgCl_2 , 100 mM additional monovalent salt, and *N*-methylglucamine (NMG) to pH 7.2. Extracellular solutions (except for **a**) contained 5 mM CaCl_2 , 5 mM KCl, 100 mM NMG, and HEPES buffer to pH 7.4. For **a**, the extracellular (pipette) solution contained no NMG and 20 mM HEPES (pH 7.4); the cytoplasmic solution contained 30 mM $\text{Cs}_4\text{-BAPTA}$, no EGTA, 25 mM HEPES buffer (pH 7.2) and 60 mM additional monovalent salt.



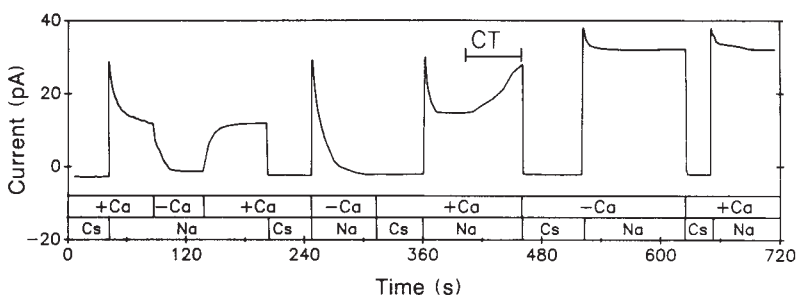
The transient nature of exchange current on activation by cytoplasmic sodium complicates the study of its ionic dependence. Figure 2 shows the dependence on cytoplasmic sodium concentration (Fig. 2*b*) and cytoplasmic calcium concentration (Fig. 2*c*) of peak and steady-state exchange currents (24 °C; no Mg-ATP applied). The current transient simply scales down with the decrease in the activating sodium concentration; by contrast, the transient fraction decreases and is abolished as cytoplasmic free calcium increases ($n > 10$; Fig. 2*a*). Half-maximal cytoplasmic sodium with 0.3 μM free cytoplasmic calcium is $\sim 15 \text{ mM}$ for both peak and steady-state currents (Fig. 2*b*). The steady-state outward current (filled circles) increases over the range of 0.1 to 20 μM free calcium, and then decreases with reduction of the driving force as cytoplasmic calcium is increased further (Fig. 2*c*). Strikingly, peak current (open circles) is activated by a log-unit-lower free calcium than is the steady-state current.

tude in the absence of calcium. This time course ($t_{1/2} \approx 5$ s) must reflect both inactivation following activation by sodium, and inhibition by removal of calcium.

In a patch from a shortened myocyte, brief chymotrypsin treatment also abolished modulation of the exchange current by Mg-ATP (Fig. 4). In the control, the current activated by sodium decreased by >80% over less than 3 s (37 °C). After application of chymotrypsin for 1.5 min, the current had doubled, and it did not decay. The current was not dependent on cytoplasmic free calcium up to the maximum of 1 μ M tested (data not shown), and superfusion of 5 mM Mg-ATP had no effect.

That chymotrypsin converts the exchanger in patches to a highly active state and abolishes secondary exchange modulation by cytoplasmic calcium and Mg-ATP, suggests that a proteolysis-susceptible inhibitory domain of the exchanger, or an auxiliary inhibitory protein, mediates secondary exchange regulation. Because excised patches retain regulatory mechanisms, whereas cardiac membrane vesicles do not, the modulator is presumably altered by vesicle isolation procedures. It is interesting that in this context, chymotrypsin has been found to remove a fragment of relative molecular mass 50,000 of the cardiac exchanger protein without loss of activity¹⁸.

Figure 1 consists of three panels. Panel (a) is a line graph of Current (pA) vs Time (s). The y-axis ranges from 0 to 30 pA, and the x-axis ranges from 0 to 40 s. A solid line shows the current response to a step from 0 Na to X mM Na, peaking at approximately 30 pA and then decaying to a steady state of about 15 pA. A dashed line shows the current response to a step from X mM Na to 0 Na, returning to 0 pA. A dotted line shows the current response to a step from 0 Na to X mM Na, peaking at approximately 30 pA and then decaying to a steady state of about 25 pA. Panel (b) is a line graph of Current (pA) vs Sodium (mM). The y-axis ranges from 0 to 20 pA, and the x-axis ranges from 0 to 100 mM. The current increases with sodium concentration, reaching a plateau of about 18 pA at 100 mM. Panel (c) is a line graph of Current (pA) vs Calcium (pCa). The y-axis ranges from 0 to 20 pA, and the x-axis ranges from 8 to 3 pCa. The current increases with calcium concentration, reaching a peak of about 20 pA at 5 pCa, and then decreases to about 8 pA at 3 pCa.



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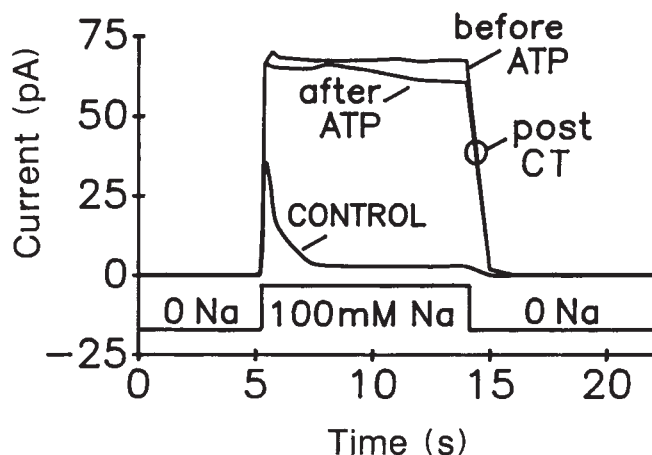


FIG. 4 Chymotrypsin removes regulation by Mg-ATP. Conditions: $0.3 \mu\text{M}$ cytoplasmic Ca^{2+} , 37°C and 0 mV . Exchange current after addition of α -chymotrypsin was doubled and did not decay (post CT). Mg-ATP had no effect.

Three observations point to a link between secondary exchange activation by cytoplasmic calcium and the apparent inactivation on elevating cytoplasmic sodium. First, the two processes have similar kinetics regardless of experimental conditions (for example, see first part of Fig. 3). Second, the activation of exchange current by cytoplasmic calcium is specifically shifted by the apparent inactivation during application of cytoplasmic sodium (Fig. 2c). Third, the two processes are abolished together by limited proteolysis (Fig. 3). If activation of outward exchange current by cytoplasmic calcium (Fig. 2c) reflects occupation of a calcium-specific regulatory site, then the affinity of regulatory sites is evidently decreased by occupation of a sodium-binding site(s). The time courses of exchange-current decay on both removal of cytoplasmic calcium and application

of cytoplasmic sodium could, therefore, reflect exchanger inhibition by the regulator entity after release of bound calcium.

The ability to 'deregulate' $\text{Na}^+-\text{Ca}^{2+}$ exchange in excised patches should allow the catalytic properties of the exchanger to be studied independently of secondary modulation. Although it is tempting to assume that a protein kinase underlies the actions of Mg-ATP, this remains to be established. I have here demonstrated the direct nature of secondary exchange activation by cytoplasmic calcium. That modulators of the exchanger are not lost from rapidly superfused excised patches raises important questions about their physical attachment, mobility and relationship to the exchanger itself. The giant patch technique should facilitate a resolution of these and related issues in the regulation of sarcolemmal electrogenic mechanisms. □

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1. Reuter, H. & Seitz, N. *J. Physiol.* **195**, 451-470 (1968).
2. Allen, T. J. A., Noble, D. & Reuter, H. (eds) *Sodium-Calcium Exchange* (Oxford University Press, 1989).
3. Hilgemann, D. W. & Noble, D. *Proc. R. Soc. B* **230**, 163-205 (1987).
4. Kimura, J., Miyama, S. & Noma, A. *J. Physiol.* **384**, 199-222 (1987).
5. Reeves, J. P. in *Intracellular Calcium Regulation* (ed. Bronner F.) 305-347 (Liss, New York, 1990).
6. Baker, P. & McNaughton, P. A. *J. Physiol.* **259**, 103-144 (1976).
7. DiPolo, R. & Beaugé, L. *Biochim. biophys. Acta* **947**, 549-569 (1988).
8. Rasgado-Flores, H., Santiago, M. & Blaustein, M. P. *J. gen. Physiol.* **93**, 1219-1242 (1989).
9. Haworth, R. A., Gorkunur, A. B., Hunter, D. R., Hegge, J. O. & Berkoff, H. A. *Circulation. Res.* **60**, 586-594 (1987).
10. Caroni, P. & Carafoli, E. *Eur. J. Biochem.* **132**, 451-460 (1983).
11. Hilgemann, D. W., *Pflügers Arch. ges. Physiol.* **415**, 247-249 (1989).
12. Hilgemann, D. W., *Prog. Biophys. molec. Biol.* **51**, 1-45 (1988).
13. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85-100 (1981).
14. Allen, T. J. A. & Baker, P. F. *J. Physiol.* **378**, 53-76 (1986).
15. Cervetto, L., Lagnado, L., Perry, R. J., Robinson, D. W. & McNaughton, P. A. *Nature* **337**, 740-743 (1989).
16. DiPolo, R. *J. gen. Physiol.* **73**, 91-113 (1977).
17. Philipson, K. D. & Nishimoto, A. Y. *Am. J. Physiol.* **243**, 191-195 (1982).
18. Philipson, K. D., Longoni, S. & Ward, R. *Biochim. biophys. Acta* **945**, 298-306 (1988).

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Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis

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THE deposition of silica particles in the lung of man or experimental animals leads to silicosis, a disease of progressive respiratory failure caused by a fibrotic reaction¹. It has long been suspected that the phagocytosis of silica by pulmonary macrophages induces the secretion of fibrogenic factors². Several potentially fibrogenic cytokines released by macrophages have been identified, including interleukin-1 (IL-1) (ref. 3), tumour necrosis factor- α (TNF) (ref. 4), platelet-derived growth factor⁵, basic fibroblast growth factor⁶ and transforming growth factor- β (TGF- β) (ref. 7). Here we show that TNF plays an important part in silica-induced pulmonary fibrosis in mice in that (1) a single instillation of silica leads to a marked increase in the level of lung TNF messenger RNA which lasts for >70 days, while there are no obvious changes in the amounts of IL-1 α or TGF- β mRNAs; and (2) silica-induced collagen deposition is almost completely prevented by anti-TNF antibody, but is significantly increased by continuous infusion of mouse recombinant TNF.

A suspension of silica particles, or solvent alone as control, was instilled intratracheally in (CBA $\times$ C57BL/10)F1 mice.

Instillation of silica particles leads to the formation of silicotic nodules which are composed mainly of macrophages and fibroblasts within a rich network of collagen fibrils⁸. After instillation, we extracted total lung RNA and determined the mRNA levels at different times of TNF, TGF- β and IL-1 α (which probably represents expression of both IL-1 α and IL-1 β genes as these are co-expressed⁹). Lung TNF mRNA was not found in control mice but was evident in some mice at day 7, in all mice at day 15 and in mice examined up to day 70 after silica instillation (for examples see Fig. 1), although no TNF was detectable in serum by the L929 bioassay¹⁰. Intravenous injection of *Escherichia coli* lipopolysaccharide as a positive control for lung IL-1 and TNF mRNAs gave a marked but transient (3 h) increase in these mRNAs (Fig. 1, lane 9). TGF- β mRNA was detectable in the lungs of control mice, but did not vary significantly after silica instillation (Fig. 1).

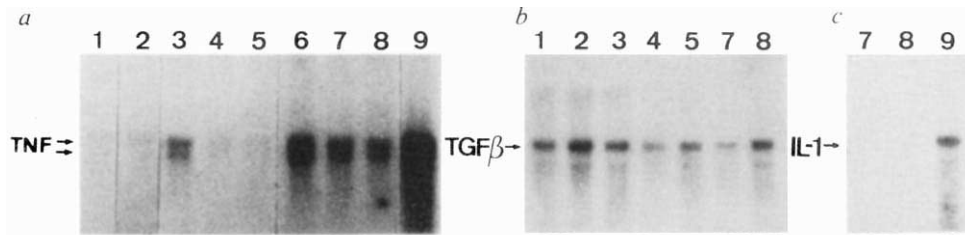
To investigate the role of TNF in the fibrotic process, we treated silica-instilled mice with rabbit anti-mouse TNF (Fig. 2b) or non-immune IgG (Fig. 2a) and killed them after two weeks. As seen in the light microscope, anti-TNF treatment does not diminish the size or number of silicotic nodules when compared with those of normal IgG-treated controls. But after silver staining, the nodules of anti-TNF treated mice are less dense and contain fewer microfibrils; this corresponds to a lack of pleural retractions resulting from parenchymal fibrosis (Fig. 2b). To quantitate the deposition of collagen, lung tissue was treated with acid and the hydroxyproline content determined¹¹. In agreement with previous observations, there was a significant increase in lung hydroxyproline 15 days after silica instillation⁸, which was completely prevented in mice treated with anti-TNF antibody (Fig. 3a). By infusing mouse recombinant TNF (or its solvent as control) through an osmotic minipump over 15 days,

we showed that exogenous TNF has little effect on the collagen content of the lungs of normal mice (Fig. 3*b*), but in silica-treated mice it significantly increases collagen deposition (Fig. 3*c*). In histological sections, the nodules of TNF-infused mice appear to be more dense, with larger collagen fibrils.

The most likely source of TNF is from the silica-containing macrophages present inside the silicotic nodule. *In situ* hybridi-

zation confirms that TNF mRNA is present in the silicotic nodules (Fig. 4). Immunohistochemical staining with anti-TNF antibody, revealed with peroxidase-labelled anti-rabbit immunoglobulin, shows that peroxidase-stained cells (not clear on black-and-white photomicrographs) are interspersed within the silicotic nodules. Interleukin-6, which is produced by several cell types on stimulation with appropriate cytokines, including

FIG. 1 Expression of TNF- α , TGF- β and IL-1 α mRNA in the lung during silicosis. A silica particle suspension (20 mg ml⁻¹; particle size <5 μ m) in saline or saline alone as a control were injected intratracheally (0.1 ml). Total lung RNA from individual mice was isolated by guanidine-thiocyanate treatment and caesium chloride centrifugation. Mouse *a*, TNF; *b*, TGF; and *c*, IL-1 mRNA levels were detected by northern blot hybridization²³. Uniformity of RNA loading (4 μ g per lane) and transfer was evaluated by methylene blue staining of the filter before hybridization, which reveals the amount of transferred 28S and 18S ribosomal RNA. Filters were hybridized with ³²P-labelled complementary RNA probes derived from plasmid pSP65-TNF containing a 1,750-bp *Eco*RI-PstI fragment²³, pSP65-IL-1 containing a 416-bp



PstI-PvuII fragment²³ and pSP65-TGF- β , containing a 1,600 bp *Eco*RI cDNA fragment²⁴. Lanes with the same number correspond to the same samples and are as follows: lanes 1 and 2, saline; lanes 3–5, silica day 7; lanes 6–8, silica day 15; lanes 9, 1 h after an intravenous injection of 100 μ g *E. coli* lipopolysaccharide.

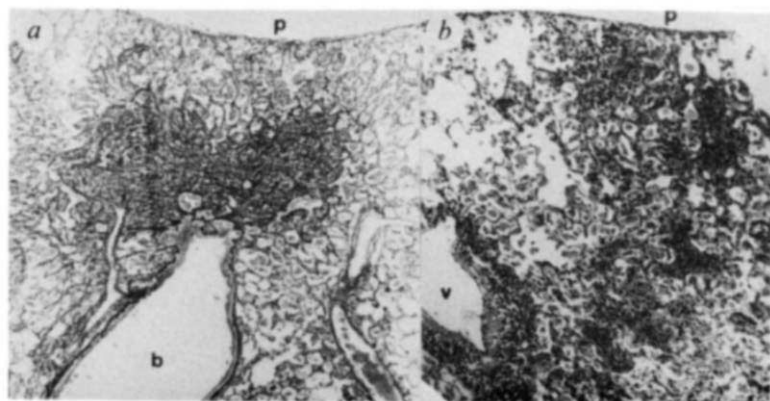


FIG. 2 Histological section (silver staining; magnification, $\times 40$) of the lungs from mice killed 15 d after instillation of silica and treatment with non-immune rabbit IgG (*a*) or anti-TNF IgG (*b*). Rabbit anti-mouse recombinant TNF or normal IgG fractions were prepared as described^{16,22} and centrifuged at 150,000g for 150 min. IgG (1 mg) was injected intraperitoneally on days 1 and 8 after silica instillation. Anti-TNF activity¹⁰ detected by the neutralization of TNF-mediated cytotoxicity in a fibroblast cell line was still detectable within the recipient's serum 5 d after injection. *a*, Silicotic formation is seen above a main bronchus (*b*); it contains numerous argentaffin fibres and has produced a retraction of the pleura (*p*). *b*, Silicotic lesions, which exist mainly as areas of filled alveolae (partly with macrophages), but without argentaffin fibres, are seen between a main blood vessel (*v*) and the pleura (*p*), which is not retracted.

FIG. 3 Influence of anti-TNF antibody and of TNF perfusion on lung hydroxyproline content. The results are the mean $\pm$ s.e.m. of the total lung hydroxyproline in mice killed 15–17 days after silica instillation. Hydroxyproline concentration was determined colourimetrically after acid hydrolysis¹¹. *a*, Rabbit anti-TNF or non-immune IgG (nIgG) were injected as described in the legend to Fig. 2; *b* and *c*, mouse *E. coli* recombinant TNF (rTNF) (specific activity 3×10^7 units mg⁻¹, as determined in a TNF bioassay¹⁰), or the control solvent, was administered by continuous intraperitoneal perfusion using an Alzet osmotic minipump (Alza, Palo Alto; number 2002) at a rate of 200 ng per hour. The non-parametric Mann-Whitney *U*-test was used for statistical analysis. Abbreviations: i.p., intraperitoneal; i.t., intratracheal.

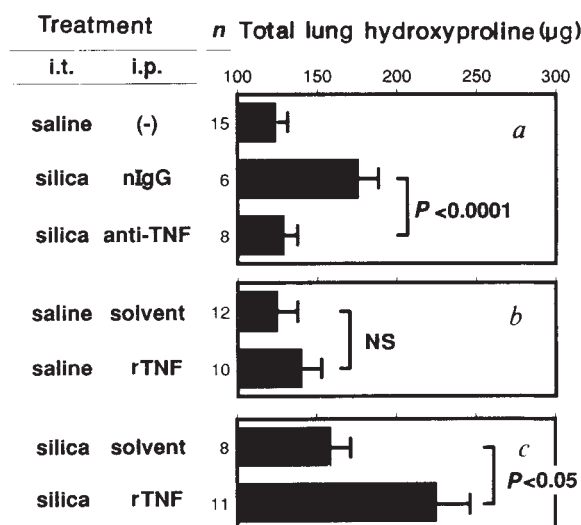


FIG. 4 Localization of TNF mRNA in silicotic nodules by *in situ* hybridization. Mice were killed 15 d after silica instillation, the lungs were instilled with 10% albumin, frozen, and cryostat sections hybridized with a ³²P-labelled TNF cRNA probe²⁵. Sections were exposed for 7 d. *a*, Haematoxylin and eosin-stained section, showing silicotic nodules, some of which are indicated by an arrow; *b*, autoradiograph of the same section showing that the grains are located over the nodules (arrows) but not over the lung parenchyma (magnification, $\times 3$).

TNF¹², is also detectable in most cells from the nodule, but not in the serum. Electron micrographs of silicotic nodules show that silica-containing macrophages are in close contact with fibroblasts (data not shown). *In vivo*, local release of TNF causes a focal accumulation of fibroblasts and collagen¹³, so the TNF released by macrophages might act directly on fibroblasts, increasing their numbers and/or rate of collagen secretion. In the same way, as TNF infusion results in alveolar damage¹⁴, TNF overproduction in the silicotic nodule might be responsible for changes in the surrounding epithelial cells¹⁵.

We have recently shown that TNF is also important in pulmonary fibrosis induced by the drug bleomycin¹⁶. But in this condition, T lymphocytes are necessary both for the increase in lung TNF mRNA and the development of fibrosis¹⁶, whereas silicosis is not markedly affected by the absence of T lymphocytes¹⁷. Furthermore, and possibly related to the T lymphocyte involvement, TGF- β and its mRNA increase during the early phase of the bleomycin pneumopathy^{18,19}, but TGF- β mRNA levels do not vary significantly during silicosis (Fig. 1). Of the potential fibrogenic factors, IL-1, which is released *in vitro* by silica-stimulated macrophages²⁰, is not overproduced during silicosis (Fig. 1); platelet-derived or basic fibroblast growth factors could possibly be involved. Therefore the effect of TNF on the development of silicosis might result from a local and sustained release of TNF at higher levels than other potential fibrogens.

Pneumoconioses are potentially killing diseases, usually incurable by available therapy. In man, silicosis is frequently associated with tuberculosis²¹. Although silicosis might favour the development of mycobacteria, the reverse could be true because BCG granuloma are a rich source of TNF²², which is likely to aggravate the fibrotic reaction. Indeed BCG vaccination

against tuberculosis can lead to an aggravation of silicosis²¹. It is worth mentioning that corticosteroids markedly decrease the transcription of the TNF gene²³. Our results indicate that modulation of cytokines that control collagen deposition and degradation could be a new approach to treatment. □

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1. Bowden, D. H. *Expl. Lung Res.* **12**, 89–107 (1987).
2. Heppleston, A. G. & Styles, J. A. *Nature* **214**, 521–522 (1967).
3. Schmidt, J. A., Mizel, S. B., Cohen, D. & Green, I. *J. Immun.* **128**, 2177–2182 (1982).
4. Sugarman, B. J., Aggarwal, B. B., Figari, I. S. & Palladino, M. A. *Science* **230**, 943–945 (1985).
5. Ross, R., Raines, E. W. & Bowen-Pope, D. F. *Cell* **46**, 155–169 (1986).
6. Folkman, J. & Klagsbrun, M. *Science* **235**, 442–447 (1987).
7. Roberts, A. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 4176–4171 (1986).
8. Dauber, J. J. H., Rossman, M. D., Pietra, G. G., Jimenez, S. A. & Daniele, R. P. *Am. J. Path.* **101**, 595–612 (1980).
9. Casey, T. W., Duncan, L. M. & Unanue, E. R. *J. Immun.* **142**, 3469–3476 (1989).
10. Ruff, M. R. & Gifford, G. E. *J. Immun.* **126**, 1671–1677 (1980).
11. Huszar, G., Maiocco, J. & Naftolin, F. *Analyt. Biochem.* **105**, 424–429 (1979).
12. Broukaert, P., Spriggs, D. R., Demetri, G., Kufe, D. W. & Fiers, W. *J. exp. Med.* **169**, 2257–2262 (1989).
13. Piguet, P. F., Grau, G. E. & Vassalli, P. *Am. J. Path.* **136**, 1990–1998 (1990).
14. Tracey, K. J. *et al. J. exp. Med.* **167**, 1211–1227 (1988).
15. Bowden, D. H. & Adamson, I. Y. R. *J. Path.* **144**, 149–161 (1984).
16. Piguet, P. F., Collart, M. A., Grau, G. E., Kapanci, Y. & Vassalli, P. *J. exp. Med.* **170**, 655–663 (1989).
17. Hubbard, A. K. *Lab. Invest.* **61**, 46–50 (1989).
18. Khalil, N., Berezney, O., Sporn, M. & Greenberg, A. *J. exp. Med.* **170**, 727–737 (1989).
19. Hoyt, D. G. & Lazo, J. S. *J. Pharmac. exp. Ther.* **246**, 765–770 (1989).
20. Schmidt, J. A., Oliver, C. N., Lepe-Zuniga, J. L., Green, I. & Gery, I. *J. clin. Invest.* **73**, 1462–1472 (1984).
21. Snider, D. E. *Am. Rev. Respir. Dis.* **118**, 455–460 (1978).
22. Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. F. & Vassalli, P. *Cell* **56**, 731–740 (1989).
23. Collart, M. A., Belin, D., Vassalli, J. D., de Kossodo, S. & Vassalli, P. *J. exp. Med.* **163**, 2113–2118 (1987).
24. Derynck, R., Jarret, J. A., Chen, E. Y. & Goeddel, D. V. *J. biol. Chem.* **261**, 4377–4379 (1986).
25. Sappino, A. P., Huarte, J., Belin, D. & Vassalli, J. D. *J. Cell Biol.* **109**, 2471–2479 (1989).

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Novel post-translational regulation of TCR expression in CD4⁺CD8⁺ thymocytes influenced by CD4

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EXPRESSION of the multicomponent T-cell antigen receptor (TCR) complex on the surface of thymocytes is developmentally controlled. Most immature CD4⁺CD8[−] 'double negative' and CD4⁺CD8⁺ 'double positive' thymocytes express either no or few TCR on their surface, and maturation to CD4⁺CD8[−] or CD4⁺CD8⁺ 'single positive' thymocytes is accompanied by a dramatic increase in the number of surface TCR complexes^{1–5}. Although the initial appearance of TCR during differentiation results from rearrangement and initiation of transcription of TCR genes in the thymus^{6–12}, the mechanisms regulating the quantitative changes in TCR expression during intrathymic differentiation are unknown. Surface TCR levels in T-hybridoma cells can be quantitatively regulated by a series of post-translational processes, including sorting to alternative intracellular compartments and degradation, which ensure that only fully and correctly assembled receptor complexes are efficiently transported to the cell surface^{13–16}. Quantitative increases in TCR expression on the surface of CD4⁺CD8⁺ thymocytes occur *in vivo* in response to anti-CD4 antibody treatment^{17,18}. Here we present evidence that immature CD4⁺CD8⁺ thymocytes normally retain and degrade in

the endoplasmic reticulum >90% of some endogenously synthesized TCR chains, and that the increased surface TCR expression on immature CD4⁺CD8⁺ thymocytes induced by anti-CD4 is due to an increase in the escape of newly synthesized receptor chains from the endoplasmic reticulum, and is not due to increases in RNA levels, translation, or assembly. Post-translational mechanisms therefore control the levels of TCR complexes on CD4⁺CD8⁺ thymocytes, and these mechanisms can be modulated by signalling through CD4 surface molecules.

As previously shown¹⁷, injection of anti-CD4 monoclonal antibody into mice causes a 3 to 4-fold increase in the number of surface TCR complexes on immature CD4⁺CD8⁺ thymocytes (Fig. 1a). Analysis of surface TCR complexes of CD4⁺CD8⁺ thymocytes revealed that *in vivo* anti-CD4 treatment results in increased surface expression of all TCR subunits (α , β , γ , δ , ϵ and ζ ; Fig. 1b). To examine whether increased surface TCR expression in CD4⁺CD8⁺ thymocytes from mice treated with anti-CD4 antibody was due to a net increase in the total number of receptors or to a redistribution of the same number of receptors between intracellular and cell-surface pools, we isolated TCR complexes from detergent-solubilized CD4⁺CD8⁺ cells from control and anti-CD4-treated mice using an anti-CD3- δ antibody, and quantitated the total amount of CD3- δ by immunoblotting (Fig. 1c). CD4⁺CD8⁺ thymocytes from anti-CD4-treated mice had more than twice (2.3 ± 0.7 , $n = 5$) as much total CD3- δ as control mice, ruling out a simple redistribution of a constant number of receptor chains. Probing of the same blots with anti- ζ antibodies revealed increased amounts of co-precipitated ζ -subunits in cells from anti-CD4-treated mice (3.6 ± 1.4 , $n = 3$), demonstrating an increased number of assembled receptor complexes (Fig. 1c).

The presence of an increased number of receptor chains and assembled receptor complexes in CD4⁺CD8⁺ thymocytes from anti-CD4-treated mice could be due to increased synthesis of receptor chains, increased assembly of receptor complexes or decreased degradation. We initially suspected that an increase

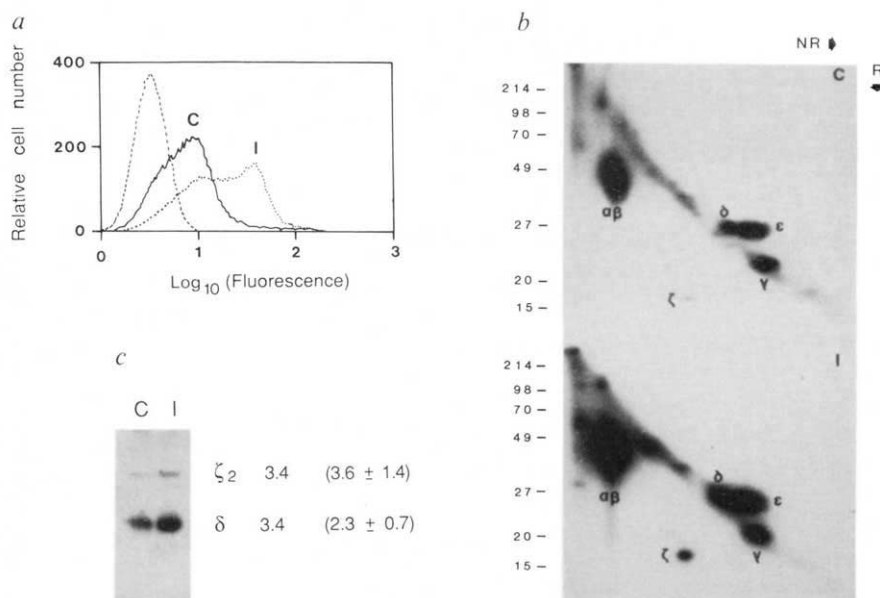
in the number of receptor chains was probably due to increased levels of TCR RNA. But measurement of RNA levels by northern-blot analysis revealed that, on the average, CD4⁺CD8⁺ thymocytes from anti-CD4-treated mice had lower steady-state levels of RNA for most of the receptor chains than did CD4⁺CD8⁺ cells from untreated mice (Fig. 2a). To analyse whether any of the RNAs were translated more efficiently on anti-CD4 treatment, we pulse-labelled purified CD4⁺CD8⁺ thymocytes with [³⁵S]-methionine, and directly isolated the different TCR chains with specific antibodies (Fig. 2b-d). These experiments showed that biosynthesis of β -, CD3- γ -, δ -, ϵ and ζ chains was either not changed or slightly decreased by the anti-CD4 treatment (Fig. 2b-d). Synthesis of α -chains could not be directly assessed because of the lack of a specific antibody to them. But analysis of anti- β immunoprecipitates by two-dimensional nonreducing-reducing SDS-PAGE revealed equal or lower amounts of $\alpha\beta$ -heterodimers in anti-CD4-treated cells (Fig. 2c). Association of the T-cell receptor-associated protein (TRAP), which binds transiently to newly synthesized CD3 chains in the endoplasmic reticulum (ER)^{19,20}, was also not increased in anti-CD4-treated thymocytes (Fig. 2b). We obtained similar results by pulse-labelling either CD4⁺CD8⁺ thymocyte cell suspension or thymus fragments (data not shown). Resolution of anti- ϵ immunoprecipitates by two-

dimensional nonreducing-reducing SDS-PAGE also showed equivalent assembly of newly synthesized TCR complexes in cells of the control and anti-CD4 groups (Fig. 2d).

The above observations demonstrate that the net increase in the total number of TCR complexes found in CD4⁺CD8⁺ thymocytes from anti-CD4-treated mice cannot be ascribed to elevated steady-state levels of TCR RNA or to increased translation or assembly of receptor chains. A decrease in the degradation of receptors remained the only explanation for the changes observed. Assessment of the degradation rate of newly synthesized receptor chains requires pulse-chase metabolic labelling of thymocytes at 37 °C over a time span of several hours, and requires TCR expression to be stable in these cells during this time. But suspension culture at 37 °C of CD4⁺CD8⁺ thymocytes from normal mice results in a spontaneous increase in surface TCR expression¹ (Fig. 3a). By contrast, we found that TCR expression on CD4⁺CD8⁺ cells remained stable *in vitro* if thymocytes were cultured as intact organ fragments consisting of small (1–2 mm) thymic pieces (Fig. 3c), indicating that the phenotype of low expression of surface TCR in CD4⁺CD8⁺ thymocytes requires an intact thymic microenvironment. Consequently, we examined the survival of newly synthesized CD3- δ subunits in control thymocytes metabolically labelled either in suspension culture or in organ fragment culture. It was striking

FIG. 1 Treatment with anti-CD4 antibody *in vivo* causes an increase in the number of TCR complexes in CD4⁺CD8⁺ thymocytes. **a**, CD4⁺CD8⁺ thymocytes from control mice injected with saline (C) or mice injected with anti-CD4 monoclonal antibody (I) were stained with either a negative control antibody (Leu 4, dashed line) or an anti-mouse CD3- ϵ antibody (145-2C11, solid and dotted lines). **b**, Structure of TCR complexes in CD4⁺CD8⁺ thymocytes from control and anti-CD4-treated mice. CD4⁺CD8⁺ thymocytes purified from control saline-injected mice (C) or from anti-CD4-injected mice (I) were surface labelled with ¹²⁵I. TCR complexes were isolated by immunoprecipitation with an anti-CD3- ϵ antibody (145-2C11) and resolved by two-dimensional nonreducing (NR)-reducing (R) SDS-PAGE. The positions of relative molecular mass (M_r) markers (expressed as $M_r \times 10^{-3}$) are shown on the left. The positions of TCR chains are indicated. **c**, Quantitation of total CD3- δ and associated ζ -chains by immunoblotting. Purified CD4⁺CD8⁺ thymocytes from control (C) or anti-CD4-injected (I) mice were extracted with 0.5% Triton X-100. TCR complexes were isolated with anti-CD3- δ Sepharose beads and separated by SDS-PAGE under nonreducing conditions. Proteins were transferred to nitrocellulose and blots were successively probed with anti- δ and anti- ζ antibodies followed by ¹²⁵I-labelled protein A. Numbers to the right of each band correspond to the I:C ratios observed for the experiment shown in this figure. Numbers in parentheses represent the mean $\pm$ s.d. of the I:C ratios observed in three and five experiments for ζ 2- and δ -subunits, respectively.

METHODS. C57BL/6 pregnant female mice were obtained from the Jackson Laboratory, Bar Harbor, Maine or from the Frederick Cancer Research Facility, Frederick, Maryland. Starting on day 7–11 after birth, mice received two or three daily intraperitoneal injections with 200 μ g purified GK 1.5 anti-CD4 monoclonal antibody²¹. One day after the last injection, thymocytes were prepared, and CD4⁺CD8⁺ cells were purified either by staining for CD8 followed by cell sorting or by panning on dishes coated with anti-CD8 monoclonal antibody (83-12-5 anti-Lyt 2.2 IgM). Cells purified by either method were > 96% CD4⁺CD8⁺ (data not shown). **a**, CD4⁺CD8⁺ thymocytes from control and anti-CD4-injected mice were prepared and stained with 145-2C11 anti-CD3 monoclonal antibody conjugated with fluorescein isothiocyanate (FITC). The negative control stain was Leu4-FITC, which is specific for human cells and does not stain murine thymocytes. Viable cells (50,000), as defined by forward light-scatter intensity and propidium iodide exclusion, were analysed for each sample on a modified FACS II (BDIS) interfaced to a PDP 11/84 computer (Digital Equipment Corp., Maynard, Massachusetts). **b**, CD4⁺CD8⁺ thymocytes purified from control and anti-CD4-injected mice (5×10^7 cells per sample) were surface iodinated with



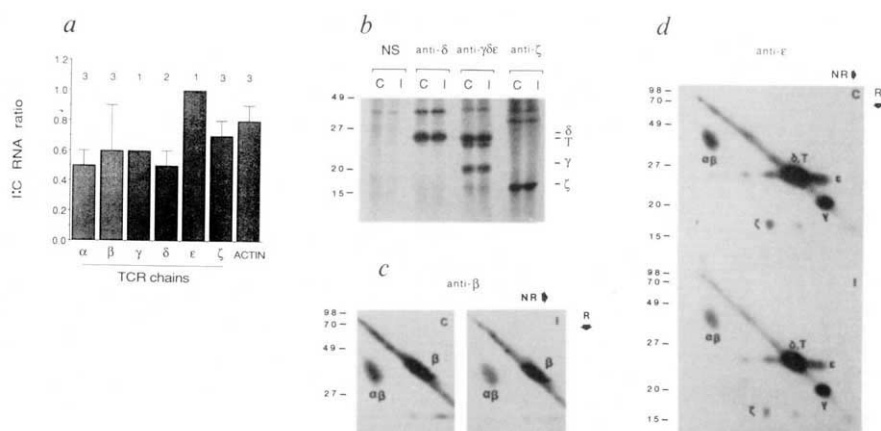
2.5 mCi ¹²⁵I using lactoperoxidase, as previously described²². The amount of ¹²⁵I incorporated was determined by precipitation with 10% TCA and was similar for both samples. Labelled cells were extracted at 4 °C with lysis buffer (0.5% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4) and ¹²⁵I-labelled TCR complexes were isolated from a fraction with an activity $\sim 1.1 \times 10^7$ c.p.m. using the 145-2C11 anti- ϵ monoclonal antibody. TCR chains were resolved by two-dimensional nonreducing-reducing SDS-PAGE, as previously described²². **c**, Purified CD4⁺CD8⁺ thymocytes (1.2×10^7 cells) from control (C) and anti-CD4-treated mice (I) were solubilized with 240 μ l lysis buffer. After removing the insoluble material, the supernatant was incubated for 1 h at 4 °C with an anti-CD3- δ polyclonal antibody (no. 1489)²³ covalently coupled to CNBr-activated Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden). After incubation, the beads were washed three times with 1 ml wash buffer (0.1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4), boiled in 80 μ l nonreducing electrophoresis sample buffer, and TCR chains separated by SDS-PAGE on 13% acrylamide gels²². After electrophoresis, proteins were electroblotted onto nitrocellulose paper according to the method of King *et al.*²⁴ and the blots were probed with a 1:200 dilution of an antisera directed against the CD3- δ chain²³ followed by ¹²⁵I-labelled protein A (10 μ Ci per blot), as previously described²⁵. After autoradiography, the blots were re-probed using an anti- ζ antiserum²⁶. Autoradiograms were analyzed by densitometric scanning.

that in either case, most newly synthesized CD3- δ chains were rapidly degraded (Fig. 4a). Such rapid degradation was not a general characteristic of proteins synthesized by CD4⁺CD8⁺ thymocytes, because the total amount of radioactivity incorporated into proteins decreased by only ~35% after 10 h of chase (data not shown). To directly determine the effect of *in vivo* anti-CD4 treatment on the relative degradation rates of TCR chains, we compared the relative survival of newly synthesized CD3- δ in organ fragment cultures of thymi from control and anti-CD4-treated mice. In three separate pulse-chase experiments, relative survival of CD3- δ chains was increased by up

to 220% in thymic fragment cultures from anti-CD4-treated mice (Fig. 4c), an amount that is sufficient to account for the increase in total receptors observed in them. Even so, we note that because the double-positive thymocytes could not be isolated in these experiments, this increase is likely to be an underestimate of the quantitative effect of anti-CD4 antibody on the responding subpopulation of cells. A similar mechanism could account for the increased TCR expression by CD4⁺CD8⁺ thymocytes placed in *in vitro* suspension cultures, because *in vivo* anti-CD4 treatment and *in vitro* suspension culturing were not additive strategies (Fig. 3b). Indeed, as was the case with anti-CD4

FIG. 2 RNA levels and biosynthesis of TCR chains in CD4⁺CD8⁺ thymocytes from control (C) and anti-CD4-treated (I) mice. *a*, Relative levels of TCR RNAs in CD4⁺CD8⁺ thymocytes from anti-CD4-injected versus control mice. Total RNA from purified CD4⁺CD8⁺ thymocytes was analysed by northern-blot hybridization using probes for α -, β -, CD3- δ -, γ -, ϵ - and ζ -chains, and actin RNAs. Results are expressed as the ratio of the amount of hybridizing specific RNA in anti-CD4-injected (I) versus control (C) mice. Values represent the mean $\pm$ s.d. of results obtained in the number of experiments indicated at the top of each column. *b-d*, Biosynthesis of TCR chains. CD4⁺CD8⁺ thymocytes were metabolically labelled for 30 min at 37 °C with [³⁵S]methionine. TCR chains were isolated from detergent-solubilized cells using a non-specific immunoprecipitation control monoclonal antibody (NS) or antibodies against TCR chains as indicated. Samples were analysed by one-dimensional SDS-PAGE under reducing conditions (*b*) or by two-dimensional nonreducing (NR)-reducing (R) SDS-PAGE (*c* and *d*). The positions of M_r markers (expressed as $M_r \times 10^{-3}$) are shown on the left of each panel. The positions of TCR chains and TRAP (T) are indicated.

METHODS. *a*, Thymocytes were obtained from either anti-CD4-injected or control C57BL/6 or C3H/HeN mice. RNA samples from CD4⁺CD8⁺ thymocytes were prepared by the guanidinium isothiocyanate-CsCl method as previously described²⁷. Equivalent amounts of total RNA were resolved in formaldehyde-1.1% agarose gels by electrophoresis at 30 V for 12 h, transferred to nitrocellulose, and hybridized with 5–30 $\times 10^8$ c.p.m. μg^{-1} ³²P-labelled probe at 42 °C in buffer containing 4 $\times$ SSC–40% formaldehyde, 10% dextran sulphate, 1 $\times$ Denhardt's solution, 50 $\mu\text{g ml}^{-1}$ denatured salmon sperm DNA. Filters were washed at a final stringency of 0.1 $\times$ SSC, between 55 and 60 °C. Filters were probed sequentially with α -, β -, CD3- δ -, ζ - and actin probes (experiments 1–3), or ϵ -, and γ -subunits and actin probes



(experiment 4). Stripping of specific hybridizations was performed by the method of Thomas²⁸. Quantitation of specific hybridized RNA was performed by densitometric analysis of exposed radiographic film. *b*, Purified CD4⁺CD8⁺ thymocytes were preincubated for 15 min at 37 °C (2.8×10^7 cells per group) in labelling medium (methionine-free RPMI 1640 containing 5% fetal bovine serum) and labelled for 30 min at 37 °C at 2.8×10^6 cells ml^{-1} with 0.5 mCi ml^{-1} [³⁵S]methionine (Tran ³⁵S-label, ICN Radiochemicals, Irvine, California) in labelling medium. Cells were then extracted with 1 ml lysis buffer (see Fig. 1) and TCR chains were isolated from aliquots of the extract using antibodies no. 1489 (anti- δ), no. 125 (anti- $\gamma\delta\epsilon$), no. 387 (anti- ζ), as indicated. The clonotypic anti-2B4- α antibody A2B4 was used as a non-specific (NS) precipitation control. *c* and *d*, Cells were metabolically labelled and TCR chains isolated essentially as described in *a*, except that labelling was performed on 5×10^7 cells at 5×10^6 cells ml^{-1} using 3.3 mCi ml^{-1} [³⁵S]methionine. Antibodies used for immunoprecipitation were F23.1 (anti- β , *c*) or 145-2C11 (anti- ϵ , *d*).

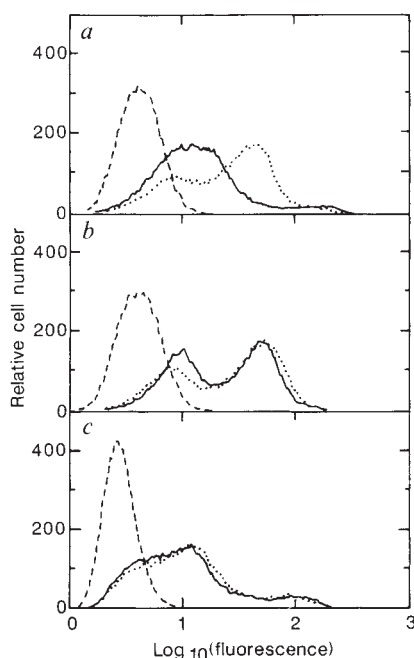
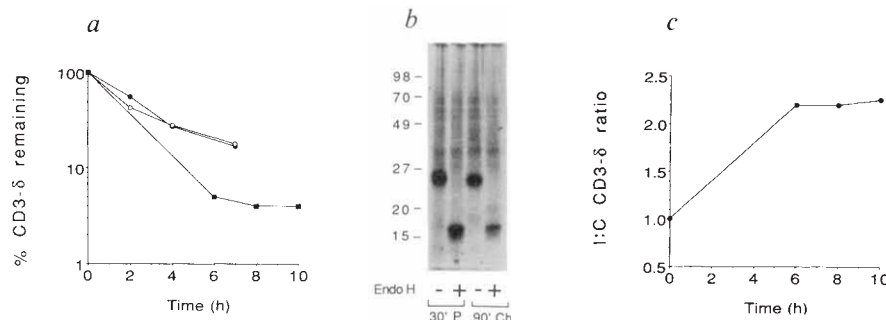


FIG. 3 CD3 expression after *in vitro* culture of thymocytes. Unseparated thymocytes from control (*a*) or anti-CD4-treated (*b*) neonatal C3H/HeN mice were cultured at 4 °C (solid lines) or 37 °C (dotted lines) and then stained with 145-2C11-FITC anti-CD3 monoclonal antibody. In a separate experiment (*c*), thymus fragments from non-injected young adult C3H/HeN mice were cultured at 4 °C (solid lines) or 37 °C (dotted lines) and then stained with 145-2C11-FITC. Dashed lines show staining of control thymocytes with a negative control monoclonal antibody (Leu4-FITC).

METHODS. *a*, Starting on day 3 after birth, C3H/HeN mice received four daily intraperitoneal injections of GK1.5 anti-CD4 monoclonal antibody. Four hours after the last injection, single cell suspensions of thymocytes were prepared and cultured (1×10^7 cells per well in 1 ml complete RPMI 1640 supplemented with 10% FCS) at 4 °C or 37 °C. After overnight culture, the cells were collected, stained and analysed as described in the legend to Fig. 1(*a*). *c*, Thymi from young adult C3H/HeN mice were cut into 1–2-mm fragments and cultured in complete RPMI 1640 medium supplemented with 10% FCS at 4 °C or 37 °C. After culture, the fragments were removed from the culture wells, and single cell suspensions were prepared from the fragments, stained and analysed as described above.

FIG. 4 Degradation of newly synthesized CD3- δ chains. *a*, Degradation of CD3- δ chains in thymocytes. Single cell suspensions of CD4⁺CD8⁺ double-positive thymocytes from untreated control mice were metabolically labelled for 30 min and chased for different periods of time in the absence (●) or the presence (○) of 20 mM NH₄Cl. Thymocytes in organ fragment cultures were similarly labelled and chased in the absence of NH₄Cl (■). CD3- δ chains were isolated by immunoprecipitation and resolved by SDS-PAGE under reducing conditions; the kinetics of degradation were examined by autoradiography and densitometry. T-hybridoma cells treated in parallel with NH₄Cl showed a significant inhibition of CD3- δ degradation by treatment with 20 mM NH₄Cl (data not shown). *b*, Lack of Golgi processing of CD3- δ chains in purified CD4⁺CD8⁺ double-positive thymocytes. Cells in suspension were pulse-labelled with [³⁵S]methionine for 30 min at 37 °C and then either placed on ice (P) or chased for 90 min at 37 °C (Ch) in complete medium. CD3- δ chains were isolated by immunoprecipitation and either not treated (–) or treated with endoglycosaminidase H (+) before analysis by SDS-PAGE under reducing conditions. Numbers on the left indicate the position of *M_r* markers (expressed as *M_r* × 10^{–3}). *c*, Survival of newly synthesized CD3- δ chains in thymocytes from control and anti-CD4-treated mice. Thymus fragments from control (C) and anti-CD4-treated (I) mice were metabolically labelled for 30 min at 37 °C with [³⁵S]methionine and chased for different periods of time at 37 °C. CD3- δ chains from an equal number of cells for each time point and condition were isolated by immunoprecipitation and resolved by SDS-PAGE. The amount of CD3- δ was quantitated by autoradiography and densitometry and expressed as the ratio of anti-CD4-treated to control cells (I:C). The experiment shown is representative of three independent experiments.



METHODS. Purified CD4⁺CD8⁺ thymocytes were preincubated for 1 h at 37 °C in regular medium in the absence or the presence of 20 mM NH₄Cl, metabolically labelled with 1 mCi ml^{–1} [³⁵S]methionine at 2 × 10⁶ cells ml^{–1} and chased for 0, 2, 4 and 7 h in complete culture medium in the absence or presence of NH₄Cl. Cells were extracted and CD3- δ chains isolated with antibody no. 1489, as described in the legend to Fig. 2. For labelling of thymus fragments, thymuses from control and anti-CD4-treated mice were cut into 1–2-mm fragments, preincubated in methionine-free medium and labelled for 30 min at 37 °C with 3 mCi ml^{–1} [³⁵S]methionine. After labelling, approximately similar amounts of thymus fragments were transferred to multi-well plates and chased in complete culture medium for 0, 6 and 8 and 10 h at 37 °C. At each time point, thymus fragments were picked up with forceps, disrupted with a glass rod and cells were counted. Equal number of cells (2.5 × 10⁶) were solubilized with lysis buffer and incorporation of [³⁵S]methionine into proteins was checked by precipitation with TCA. CD3- δ chains were isolated by immunoprecipitation with antibody no. 1489. Endoglycosaminidase-H digestion of CD3- δ chains was performed as previously described¹⁵.

treatment, a significantly greater fraction of newly synthesized CD3- δ chains survived in control CD4⁺CD8⁺ thymocytes placed in suspension cultures as compared to organ fragment cultures (Fig. 4a).

Rapid degradation of newly synthesized chains of the TCR can occur either in lysosomes or in the ER, with at least three characteristics distinguishing ER from lysosomal degradation: (1) ER degradation is insensitive to lysosomotropic agents^{15,16}; (2) the glycoprotein chains are not processed by Golgi enzymes before degradation^{15,16}; and (3) only certain components of the TCR complex (α -, β -, δ -, but not γ -, ϵ -, and ζ -subunits) are rapidly degraded^{15,16}, whereas in lysosomes α -, β -, γ -, δ - and ϵ -subunits are all degraded at similar rates^{13–16}. We therefore examined the phenotype of the rapid degradation of TCR chains in thymocytes and found that the pathway was distinctive of the ER degradation pathway. First, degradation of both δ - (Fig. 4a) and β - (data not shown) subunits were insensitive to NH₄Cl, which inhibits lysosomal degradation. Second, most of δ - (Fig. 4b) and β - (data not shown) subunits had not reached the Golgi system, as determined by endo glycosaminidase-H sensitivity of carbohydrate side chains. Third, degradation showed the characteristic selectivity of the ER pathway, in that δ - (Fig. 4a) and β - (data not shown) subunits were rapidly lost, whereas the ϵ -subunit was relatively long-lived (survival in suspension culture at 8 h was 14% for the δ -subunit, 9% for the β -subunit and 51% for the ϵ -subunit).

The regulated surface expression of TCR represents one of the characteristic phenotypes of thymic development. The results presented in this paper demonstrate a novel regulatory mechanism for the quantitative control of surface TCR numbers by immature CD4⁺CD8⁺ thymocytes. On exposure to anti-CD4 *in vivo*, CD4⁺CD8⁺ thymocytes respond with increases in both surface and total levels of the TCR complex. So far, this *in vivo* effect of anti-CD4 antibody is unique and has not been reproduced with antibodies directed at other molecules expressed on the surface of CD4⁺CD8⁺ cells. The ability to apparently reproduce the *in vivo* effect of anti-CD4 antibody by removal of CD4⁺CD8⁺ cells from the thymus could indicate that an interaction with CD4 in the thymus is necessary for maintenance of

the phenotype of low receptor number, a possibility consistent with previous observations that monovalent anti-CD4 is also effective in upregulating TCR expression¹⁸. Our present results indicate that increases in TCR number can be caused by increased survival of newly synthesized receptor chains. This regulation is superimposed on a dramatic level of destruction of newly synthesized receptor within the ER in these thymocytes, and cannot be attributed to changes in the level of TCR components made. The fate of newly synthesized TCR chains in CD4⁺CD8⁺ thymocytes represents a post-translational regulatory mechanism that is quite distinct from that observed in T-hybridoma cells, in which partially assembled TCR complexes are targeted to and degraded in lysosomes, but assembled TCR complexes are efficiently transported to the cell surface^{13,14}. We do not yet know the underlying mechanism causing the retention and degradation of these receptors in the ER. Perhaps the T cell synthesizes a transport protein that is required for exit from the ER. Regulation of the amount or function of such a molecule could provide the control site. Whether or not the further increase in surface TCR seen when double-positive thymocytes mature to single-positive T cells uses this post-translational control or requires other mechanisms remains to be determined. In either event, the ability to regulate the transport of proteins within the ER could prove to be critical for the developmentally regulated control of gene expression. □

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- Roehm, N. *et al.* *Cell* **38**, 577–584 (1984).
- Lanier, L. L., Allison, J. P. & Phillips, J. H. *J. Immunol.* **137**, 2501–2507 (1986).
- Bluestone, J. A., Pardoll, D., Sharrow, S. O. & Fowlkes, B. J. *Nature* **326**, 82–84 (1987).
- Crispe, I. N. *et al.* *J. Immunol.* **139**, 3585–3589 (1987).
- Havran, W. L. *et al.* *Nature* **330**, 170–173 (1987).
- Samelson, L. E. *et al.* *Nature* **315**, 765–768 (1985).
- Snodgrass, H. R., Dembic, Z., Steinmetz, M. & von Boehmer, H. *Nature* **315**, 232–233 (1985).
- Royer, H. D., Ramarli, D., Acuto, O., Campen, T. J. & Reinherz, E. L. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5510–5514 (1985).
- Collins, M. K. L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **82**, 4503–4507 (1985).
- Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103–107 (1985).
- Furley, A. J. *et al.* *Cell* **46**, 75–87 (1986).
- Van Dongen, J. M. *et al.* *J. Immunol.* **138**, 1260–1269 (1987).

13. Minami, Y., Weissman, A. M., Samelson, L. E. & Klausner, R. D. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2688-2692 (1987).
14. Sussman, J. J. *et al. Cell* **52**, 85-95 (1988).
15. Chen, C., Bonifacio, J. S., Yuan, L. & Klausner, R. D. *J. Cell Biol.* **107**, 2149-2161 (1988).
16. Bonifacio, J. S., Suzuki, C. K., Lippincott-Schwartz, J., Weissman, A. M. & Klausner, R. D. *J. Cell Biol.* **109**, 73-83 (1989).
17. McCarthy, S. A., Kruisbeek, A. M., Uppenkamp, I. K., Sharrow, S. O. & Singer, A. *Nature* **336**, 76-79 (1988).
18. Zuniga-Pflucker, J. C. *et al. J. exp. Med.* **169**, 2085-2096 (1989).
19. Bonifacio, J. S. *et al. J. Biol. Chem.* **263**, 8965-8971 (1988).
20. Pettey, C. L., Alarcon, B., Malin, R., Weinberg, K. & Terhorst, C. *J. Biol. Chem.* **262**, 4854-4859 (1987).
21. Dialynas, D. P. *et al. J. Immun.* **131**, 2445-2451 (1983).
22. Samelson, L. E., Harford, J. B. & Klausner, R. D. *Cell* **43**, 223-231 (1985).

23. Samelson, L. E., Weissman, A. M., Robey, F. A., Berkower, I. & Klausner, R. D. *J. Immun.* **137**, 3254-3258 (1986).
24. King, S. M., Otter, T. & Witman, G. B. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4717-4721 (1985).
25. Baniyash, M., Garcia-Morales, P., Bonifacio, J. S., Samelson, L. E. & Klausner, R. D. *J. Biol. Chem.* **263**, 9874-9878 (1988).
26. Orlloff, D. G., Frank, S. F., Robey, F. A., Weissman, A. M. & Klausner, R. D. *J. Biol. Chem.* **264**, 14812-14817 (1989).
27. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
28. Thomas, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).

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Acute leukaemia in *bcr/abl* transgenic mice

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THE Philadelphia chromosome, widely implicated in human leukaemia, is the result of a reciprocal translocation $t(9;22)(q34;q11)$ in which the *abl* oncogene located at 9q34 is translocated to chromosome 22q11, where it is fused head-to-tail with 5' exons of the *bcr* gene¹⁻⁸. In acute lymphoblastic leukaemia, some patients have a breakpoint within the major breakpoint cluster region of the *bcr* gene, whereas others have the break within its first intron^{6,9-13}. This second type of translocation results in the transcription of a 7.0-kilobase chimaeric *bcr/abl* messenger RNA translated into a *bcr/abl* fusion protein, p190, which has an abnormal tyrosine kinase activity and is strongly autophosphorylated in

TABLE 1 Combined molecular, haematological and pathological findings in *bcr/abl* transgenic mice

Animal	Transgene copy number	Age at death (days)*	WBC at death ($\times 10^9/l$)†	Diagnosis
418	7	10	ND	CML, blast crisis
536	4	13	ND	CML, blast crisis
530	8	12	ND	ALL
509	8	21	137	ALL
510	2	28	40	ALL
538	2	38	196	ALL
529	3	43	347	ALL
528	10	58	2.9	ALL/LL
523	1	alive	NA‡	NA
540	1	alive	NA‡	NA

Abbreviations: WBC, peripheral white blood cell count; ND, not determined; NA, not applicable; CML, chronic myelogenous leukaemia; ALL, acute lymphoblastic leukaemia; LL, lymphoblastic lymphoma.

* Animals 418, 536 and 530 were found dead; animals 509, 510, 528, 529 and 538 were killed when moribund.

† Preterminal WBCs. WBC of animal 510 ($\times 10^9/l$), 22.5 on day 21 and 47.5 on day 25; WBC of animal 529 ($\times 10^9/l$), 13.2 on day 28 and 297 on day 41; WBC of animal 538 ($\times 10^9/l$), 14.1 on day 18, 20.1 on day 24 and 29.8 on day 37; WBC of animal 528 ($\times 10^9/l$), 18.1 on day 28, 12.8 on day 41.

‡ Although mouse 523 and 540 are alive with normal WBC counts, a small but significant percentage (3-5%) of peripheral blood white cells were lymphoblasts.

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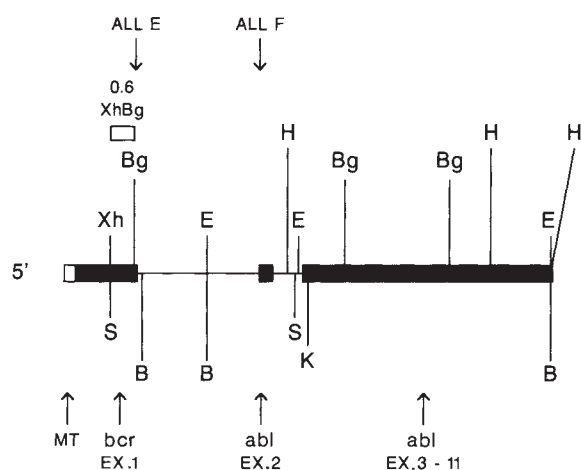


FIG. 1 p190-producing *bcr/abl* construct. Boxed areas indicate the position of coding sequences; the location of the metallothionein (MT) promoter, *bcr* exon 1, *abl* exon 2 and the main body of *abl* exons is as indicated below the map. The location of the probe used in tail blots (0.6 XhBg) is indicated above the map with a cross-hatched box. The arrows above the map point to the approximate locations of oligonucleotide primers used for the polymerase chain reaction for the reactions 'ALL E' and 'ALL F' (ref. 17). B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; S, SalI; Xh, XhoI.

METHODS. A detailed description of the construction of p190/MT will be described elsewhere. DNA fragments were purified by electrophoresis on low-melting-point agarose gels, phenol/sodium acetate extraction²⁹ and passage over an elutip-D (Schleicher and Schuell) column. Microinjections into one-cell fertilized eggs of C57Bl/CBA F1 matings were performed essentially as described³⁰.

*vitro*¹⁴. We have generated mice transgenic for a *bcr/abl* p190 DNA construct and find that progeny are either moribund with, or die of acute leukaemia (myeloid or lymphoid) 10-58 days after birth. This finding is evidence for a causal relationship between the Philadelphia chromosome and human leukaemia.

Although the presence of the Philadelphia chromosome is used as a diagnostic marker, very little is known about the direct role, if any, of *bcr/abl* in the pathogenesis of leukaemia. *In vivo*

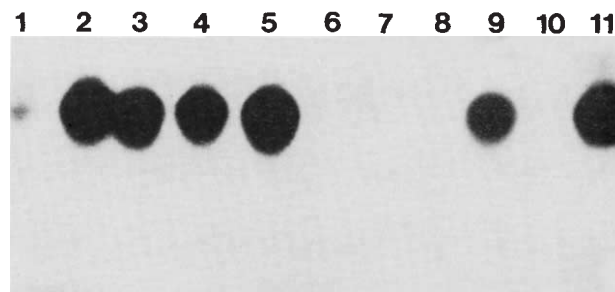


FIG. 2 Expression of the p190 *bcr/abl* construct. RNAs used include those from heart, muscle, brain and spleen of mouse 509 (lanes 1-4); NIH 3T3 cells transfected with p190/MT (lane 5); control NIH 3T3 cells (lanes 6 and 8); control non-transgenic mouse 417 RNA (lanes 7 and 10); NIH 3T3 cells transfected with p190/*bcr* (lane 9); and RNA from transgenic mouse 418 (lane 11).

METHODS. RNAs were isolated using guanidine-thiocyanate³¹. The reverse transcriptase/polymerase chain reactions were performed essentially as described¹⁷.

the translocation probably occurs in a haematopoietic stem cell, so an *in vivo* model would be useful for studying the effects of activated *bcr/abl* protein in haematopoietic cells. Expression of a *bcr/abl* construct under control of the *bcr* gene promoter is lethal during embryogenesis in transgenic mice (N.H., G.J., D. Kioussis, P.K.P. and J.G., manuscript in preparation). The

mouse metallothionein-1 promoter was chosen as an alternative promoter for the p190 *bcr/abl* construct because it can be considered as 'housekeeping' in that it is expressed in nearly all tissues¹⁵. The construct (Fig. 1) contains a segment of the metallothionein promoter, nucleotides -153 to +68 (ref. 16), joined to the first exon of the *bcr* gene. On transfection, the

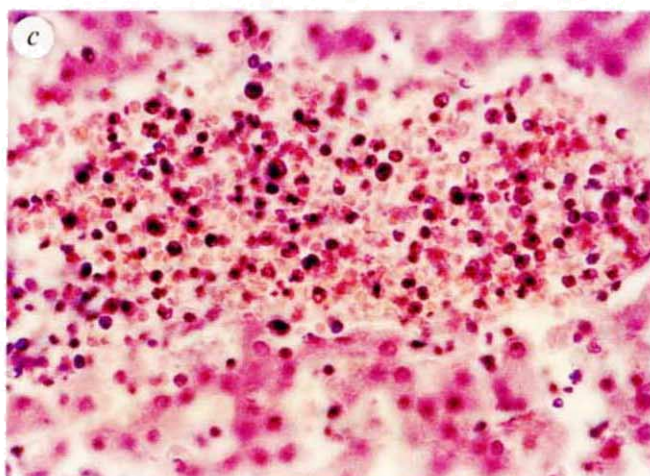
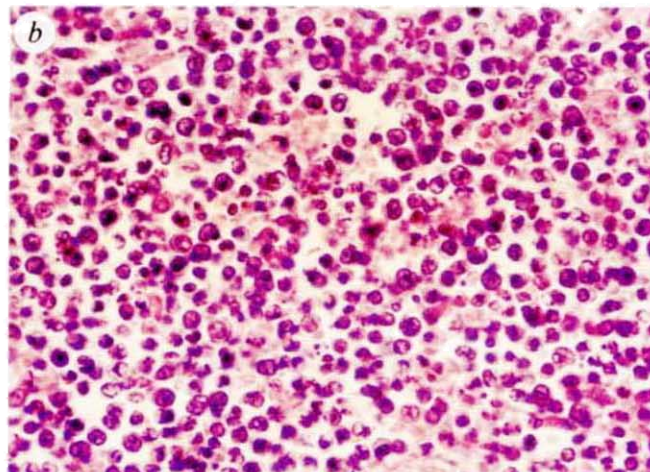
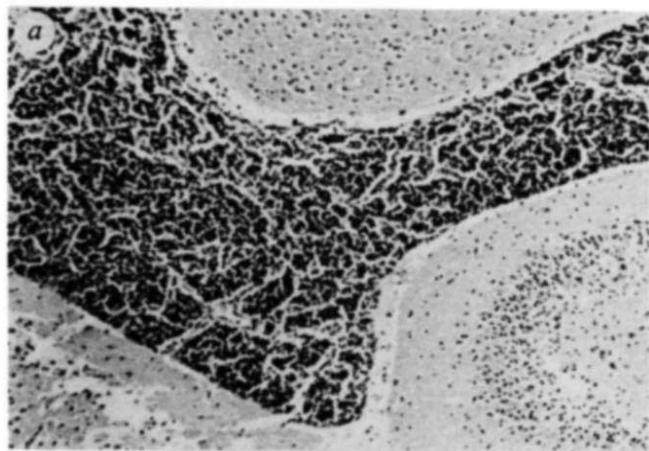


FIG. 3 Pathology of myeloid leukaemia mice. *a*, Central nervous system of mouse 536. Note the extensive tumour involvement in the subarachnoid space of the brain ($\times 120$; haematoxylin and eosin stain). *b*, Medium-power view of soft tissue-associated tumour in mouse 418. This greatly enlarged neck mass proved histopathologically to be either lymph nodes and/or subcutaneous tissue. Note the total involvement of soft tissue with neoplastic myeloid cells, which are predominantly composed of myeloblasts and promyelocytes with varying lesser numbers of myelocytes and meta-myelocytes ($\times 640$, haematoxylin and eosin stain). These morphological findings were also observed in bone marrow. *c*, Medium-power view of leukaemic peripheral blood in the hepatic vein of liver in mouse 418. Note the extensive involvement of peripheral blood with leukaemic myeloid cells, which are predominantly composed of segmented polymorphonuclear neutrophils and lesser numbers of more undifferentiated forms ($\times 640$, haematoxylin and eosin stain).

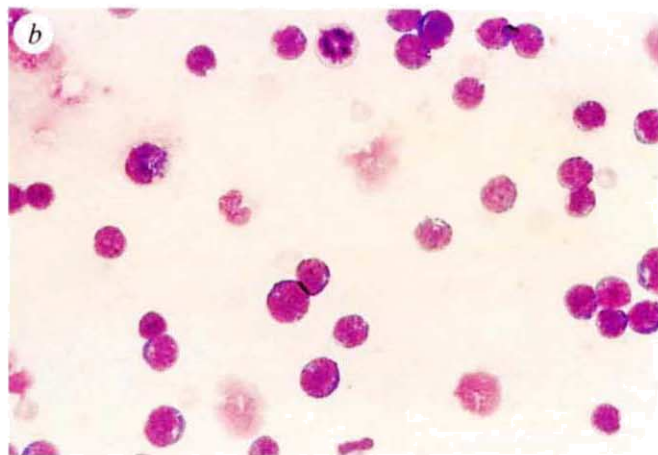
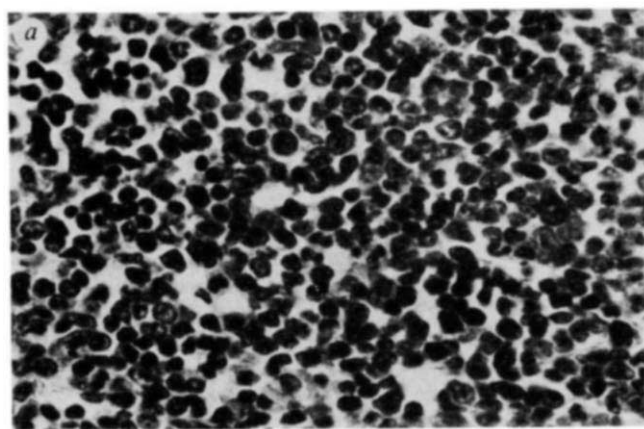


FIG. 4 Pathology of acute lymphoblastic leukaemic mice. *a*, High-power view of bone marrow from mouse 509. Note the extensive involvement of marrow with primitive lymphoblasts with scant cytoplasm ($\times 800$, haematoxylin and eosin stain). In addition, all six mice demonstrated peritubercular invasion

of neoplastic lymphoblasts into the surrounding skeletal muscle-containing soft tissues. *b*, High-power view of the peripheral blood from mouse 538. Note the presence of numerous primitive neoplastic lymphoblasts as well as a mitotic figure ($\times 800$, Wright-Giemsa stain).

construct produces a p190 protein active in an *in vitro* autophosphorylation assay (data not shown). We obtained a total of 60 progeny mice with p190/MT, 10 of which (16.6%) were transgenic by Southern blot analysis of tail DNA (data not shown). Estimated transgene copy numbers varied from 1 to 10 (Table 1).

We isolated RNAs from two animals (denoted by numbers 418 and 509) with distinct pathological symptoms (see below) to evaluate expression of the transgene using reverse transcriptase and the polymerase chain reaction¹⁷. RNA was isolated post-mortem from the leg (including marrow, bone, blood and muscle) of animal 418. The oligonucleotide primers (Fig. 1) should amplify a complementary DNA fragment of 307 base pairs in cells containing the 7.0-kilobase (kb) *bcr/abl* chimaeric mRNA. This fragment was detected in RNA isolated from NIH 3T3 cells transfected with the p190/MT construct and from an original p190 construct driven by the *bcr* promoter (Fig. 2, lanes 5 and 9), but was not found in untransfected NIH 3T3 control RNA (lanes 6 and 8). This indicates that the construct produces mRNA in the absence of intentional induction of the promoter with heavy metals. In the mouse 418 RNA, the 307-base pair fragment is also present (lane 11), but it is absent from control RNA isolated post-mortem from a non-transgenic sibling (number 417; lanes 7 and 10). Various tissue RNAs of mouse 509 were all expressed (lanes 1–4).

To date, eight of the ten transgenic animals have died, of which three (numbers 418, 536 and 530) of 10–13 days old were found dead before peripheral white blood cells could be counted (Table 1). Four animals (numbers 509, 510, 538 and 529) became progressively moribund and were killed at ages ranging from 21–43 days. Remarkably, terminal white blood cell counts in these animals ranged from $40\text{--}347 \times 10^9$ per litre (Table 1). The remaining animal was a pregnant female (mouse 528), which died at 58 days while giving birth.

Two animals (418 and 536) were diagnosed on autopsy as having myeloid leukaemia with varying degrees of differentiation towards neutrophilic granulocytes (animal 418; Fig. 3b, c). The bone marrow of mice 536 and 418 was morphologically similar and there was also marked infiltration of the central nervous system (Fig. 3a). There was a strong resemblance in these mice to the blast crisis phase (myeloid type) of chronic myelogenous leukaemia, but this diagnosis could not be confirmed as the mice were not studied prospectively. Acute lymphoblastic leukaemia was diagnosed in six mice (numbers 509, 530, 510, 538, 529 and 528) (Fig. 4). Both the splenic white and red pulp were extensively (>75%) involved with neoplastic lymphoblasts in mice 509, 510, 538 and 529. Only mouse 528 had lymph node involvement, and it was diagnosed as having acute lymphoblastic leukaemia/lymphoblastic lymphoma. There was no thymus involvement found in any of these animals.

Neoplastic lymphoblasts in frozen spleen sections of animals 509 and 510 immunostained positively for the B220 antigen. As the B220 antigen is normally found on mouse pre-B cells and on more mature B cells¹⁸, it was important to establish whether the B220⁺ lymphoblasts had undergone clonal immunoglobulin rearrangement(s). Southern blot analysis of DNA from the spleens of mice 509, 510 and 538 demonstrated a germ-line configuration for both the immunoglobulin heavy chain and kappa light chain genes (data not shown); for mouse 529, the presence of a minor clone of pre-B cells was evident. The T-cell antigen receptor β -chain locus had a germ-line configuration in all four animals. In summary, preliminary phenotypic and/or genotypic evaluation of the acute lymphoblastic leukaemic mice is consistent with the presence of a polyclonal population of neoplastic pre-B lymphoblasts, but it cannot be excluded that there is a clonal leukaemic population of lymphoid cells in these mice earlier in the B-cell differentiation process than pre-B cells.

Two types of acute leukaemia (lymphoid and myeloid) developed in our *bcr/abl* transgenic mice. In man and mouse

there is thought to be a close relationship between B-lymphoid and myeloid cells: human chronic myelogenous leukaemia often evolves to a pre-B lymphoblast crisis¹⁹, and haematopoietic cells transformed *in vitro* by *ras*-containing retroviruses co-express pre-B and myeloid cell phenotypes²⁰. There is *in vitro* evidence for a common progenitor cell, termed pro G/M/B (refs 21, 22), and such a progenitor might be affected by the *bcr/abl* p190 protein.

Most paediatric patients with Philadelphia-positive acute lymphoblastic leukaemia are pre-B in immunotype and seem to have mainly the translocation that produces p190; these patients, like adults, have a much poorer prognosis than those lacking the Philadelphia chromosome^{13,23–25}. In mouse bone marrow cultures, p190 stimulates the growth of immature lymphoid cells more than p210 (ref. 26). Our *in vivo* experiments indicate that p190 has a role in a very aggressive leukaemia with early onset and rapid progression. The rapid induction time and the polyclonality within the presumed pre-B cell population indicate that a secondary event is not necessary to generate overt leukaemia. But on the basis of theoretical models and other experimental data, acute lymphoblastic leukaemia is thought to arise after at least two independent mutations (for a discussion, see refs 27 and 28). More experiments will therefore be necessary to determine the other factors, if any, that have contributed to the rapid leukaemia in our mice.

In conclusion, we have generated ten transgenic *bcr/abl* p190 mice, eight of which died or were moribund with acute leukaemia, both myeloid and lymphoblastic, between 10 and 58 days after birth. In addition to this rapid onset, neither type of leukaemia could be distinguished from the human disease. These findings confirm the association of the Philadelphia chromosome with leukaemia in man. The availability of a mouse model for Philadelphia-positive leukaemia will improve our understanding of this disease and will allow the rapid evaluation of therapeutic protocols. □

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- Nowell, P. C. & Hungerford, D. A. *Science* **132**, 1497–1499 (1960).
- Rowley, J. D. *Nature* **243**, 290–293 (1973).
- de Klein, A. *et al. Nature* **300**, 765–767 (1982).
- Sandberg, A., Kohno, S., Wake, N. & Minowada, J. *Cancer Genet. Cytogenet.* **2**, 145–174 (1980).
- Priest, J. R. *et al. Blood* **56**, 15–22 (1980).
- de Klein, A. *et al. Blood* **68**, 1369–1375 (1986).
- Heisterkamp, N., Stam, K., Groffen, J., de Klein, A. & Grosfeld, G. *Nature* **315**, 758–761 (1985).
- Shtivelman, E., Lifshitz, B., Gale, R. B. & Canaani, E. *Nature* **315**, 550–554 (1985).
- Rodenhuis, S., Smets, L. A., Slater, R. M., Behrendt, H. & Veerman, A. J. P. *New Engl. J. Med.* **313**, 51–52 (1985).
- Erikson, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 1807–1811 (1986).
- Heisterkamp, N., Knoppel, E. & Groffen, J. *Nucleic Acids Res.* **16**, 10069–10081 (1988).
- Hermans, A. *et al. Cell* **51**, 33–40 (1987).
- Heisterkamp, N. *et al. Blood* **73**, 1307–1311 (1989).
- Kurzrock, R., Gutterman, J. U. & Talpas, M. *New Engl. J. Med.* **319**, 990–998 (1988).
- Palmiter, R., Norstedt, G., Gelinas, R. E., Hammer, R. E. & Brinster, R. L. *Science* **222**, 809–814 (1983).
- Stuart, G. W., Searle, P. F., Chen, H. Y., Brinster, R. L. & Palmiter, R. D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7318–7322 (1984).
- Kawasaki, E. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 5698–5702 (1988).
- Coffman, R. L. *Immunol. Rev.* **69**, 5–23 (1983).
- Hariharan, I. K. *et al. Molec. cell. Biol.* **9**, 2798–2805 (1989).
- Holmes, W. L., Pierce, W. F., Davidson, W. F. & Morse III, H. C. *J. exp. Med.* **164**, 443–457 (1986).
- Davidson, W. F., Pierce, J. H., Rudikoff, S. & Morse III, H. C. *J. exp. Med.* **168**, 389–407 (1988).
- Hanecak, R., Zovich, D. C., Pattengale, P. K. & Fan, H. *Molec. cell. Biol.* **9**, 2264–2268 (1989).
- Ribeiro, R. C. *et al. Blood* **70**, 948–951 (1987).
- Rivera G. K., Santana, V. M., Pui, C.-H., Furman, W. L. & Kalwinsky, D. K. in *Acute Lymphoblastic Leukemia* (eds Gale, R. P. & Hoelzer, D.) 143–146 (Wiley-Liss, New York, 1989).
- Bloomfield, C. D. *et al. in Acute Lymphoblastic Leukemia* (eds Gale, R. P. & Hoelzer, D.) 101–110 (Wiley-Liss, New York, 1989).
- McLaughlin, J., Chianese, E. & Witte, O. N. *Molec. cell. Biol.* **9**, 1866–1874 (1989).
- Greaves, M. F. in *Acute Lymphoblastic Leukemia* (eds Gale, R. P. & Hoelzer, D.) 1–14 (Wiley-Liss, New York, 1989).
- Grosfeld, G. in *Acute Lymphoblastic Leukemia* (eds Gale, R. P. & Hoelzer, D.) 15–28 (Wiley-Liss, New York, 1989).
- Heisterkamp, N., Groffen, J. & Stephenson, J. R. *J. appl. Genet.* **2**, 57–68 (1983).
- Hogan, B., Constantini, F. & Lacy, E. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, New York, 1986).
- Chomczynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156–159 (1987).

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Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins

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OXIDIZED lipoproteins have been identified in atherosclerotic plaques^{1,2} and in early lesions^{3,4} in humans as well as in animals. There is accumulating evidence that such oxidized lipoproteins have an important role in atherosclerosis⁵⁻⁷. Treatment of endothelial cells with altered lipoproteins stimulates monocyte binding⁸ as well as the production of chemotactic factors for monocytes⁹. Both these findings could be relevant to the accumulation of monocytes-macrophages in the arterial wall during the early stages of lesion development. We now report that treatment of endothelial cells (EC) with modified low-density lipoproteins obtained by mild iron oxidation or by prolonged storage, results in a rapid and large induction of the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF) and granulocyte CSF (G-CSF). These growth factors affect the differentiation, survival, proliferation, migration and metabolism of macrophages/granulocytes^{10,11}, and G-CSF and GM-CSF also affect the migration and proliferation of EC¹². Because EC and macrophages are important in the development of atherosclerosis, the expression of the CSFs by these cells could contribute to the disease.

Brief exposure of low-density lipoprotein (LDL) to Fe²⁺ or prolonged storage of isolated LDL at 4 °C results in mild oxidation corresponding to ~5 nmol thiobarbituric acid-reactive substance (TBARS) and 10 µg of cholesterol epoxide per mg LDL cholesterol. Unlike highly oxidized LDL preparations, such

minimally modified LDL (MM-LDL) is taken up by the LDL receptor rather than by the scavenger receptor⁸. Treatment of human aortic EC (HAEC) or rabbit aortic EC (RAEC) in culture with low levels (2–20 µg ml⁻¹) of MM-LDL, obtained either by iron oxidation or storage at 4 °C, resulted in dramatic induction of messenger RNA for GM-CSF, M-CSF, and G-CSF, whereas before oxidation, the same LDL preparations failed to stimulate CSF expression (Figs 1 and 2, and Table 1). For example, Fig. 1 shows results obtained using several separate preparations of LDL before and after oxidation. Altogether, more than 12 separate MM-LDL preparations yielded similar results. Also, three independent isolates of RAEC and five isolates of HAEC at a variety of passages gave comparable levels of induction, although there were reproducible differences in the basal levels of CSF expression between rabbit and human EC (Fig. 2, and Table 1). The EC cultures were essentially free of contaminating cells as judged by staining with EC-specific antibodies¹³.

Bacterial lipopolysaccharides (LPS) are also potent inducers of CSFs, and therefore it was important to rule out LPS contamination of our LDL preparations. Immunoassays indicated that the level of LPS was well below that required for CSF induction¹⁴ (Table 1). Also, the biological effects of MM-LDL and LPS were significantly different. For example, MM-LDL failed to induce interleukin-1-β (IL-1-β) expression, whereas LPS is a potent inducer of IL-1-β in vascular EC¹⁵. Also, MM-LDL induced monocyte but not neutrophil binding to EC whereas LPS induced both monocyte and neutrophil binding⁸.

The time course for the induction was similar for the three CSFs. Accumulation of CSF mRNA was apparent within 1 h of exposure to MM-LDL, peaked at ~4 h and then decreased (Table 1, data not shown). This time course is similar to that observed using other inducers of endothelial cell CSFs, including IL-1, tumour necrosis factor and LPS¹⁴. Therefore it is possible that MM-LDL and the other inducers share a signalling pathway for CSF induction.

The induction resulted in a marked increase in the secretion of biologically active CSFs into the culture medium. For example, 4 h after treatment of HAEC with MM-LDL, M-CSF activity in the medium increased tenfold as judged by a mouse bone marrow colony formation assay, and additional accumulation of M-CSF activity was observed after 24 h (Fig. 3a). Similarly, treatment of HAEC with MM-LDL for 4 h resulted in an increase in the secretion of GM-CSF activity into the medium as judged by the ability of neutralizing antibody to human GM-CSF to inhibit formation of granulocyte and macrophage colonies in a human bone marrow assay (data not shown).

We observed striking differences in the response of different

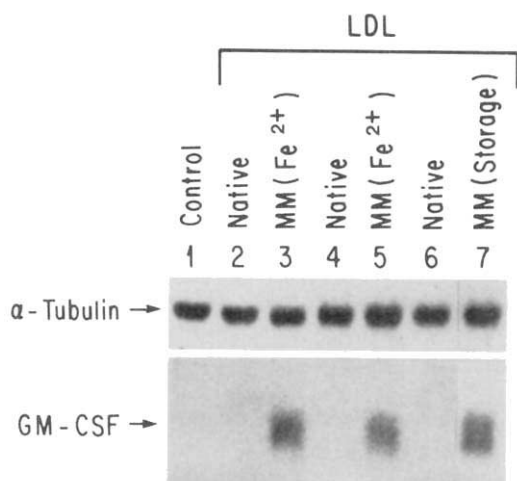


FIG. 1 Induction of GM-CSF mRNA in human aortic endothelial cells (HAEC) in response to treatment with Fe²⁺-oxidized LDL or stored LDL. Primary cultures of HAEC (passages 7–10) were treated for 4 h with various human LDL preparations (20 µg ml⁻¹). Total cellular RNA was isolated and analysed by northern blotting for levels of GM-CSF and α-tubulin mRNA. RNA was isolated from untreated cells (lane 1) or cells treated with two separate preparations of LDL before and after oxidation by mild exposure to Fe²⁺ (lanes 2 and 3, and 4 and 5), a separate LDL preparation (lane 6), and LDL oxidized by storage at 4 °C for 4 months (lane 7).

METHODS. The oxidation of LDL and treatment of EC cells were performed essentially as previously described^{8,24,25}. Total cellular RNA was isolated by lysis of endothelial cells in guanidinium isothiocyanate, phenol-chloroform extraction and precipitation²⁶. Each RNA preparation (10 µg) was denatured and electrophoresed through a 1.2% formaldehyde agarose gel followed by blotting onto nylon filters and ultraviolet crosslinking²⁷. Filters were prehybridized in 7% SDS, 0.5 M sodium phosphate, 1 mM EDTA, 1% BSA, pH 7.2. Hybridizations were performed in the same buffer for 16 h at 65 °C with an isolated GM-CSF cDNA probe²⁸ labelled to a specific activity of ~10⁸ c.p.m. µg⁻¹ (ref. 29). The blots were washed in 0.5 × SSC, 0.1% SDS at 65 °C and autoradiographed overnight. The blots were then rehybridized with an α-tubulin cDNA probe³⁰ as an internal control.

endothelial cell types. Whereas aortic EC from both humans and rabbits were highly responsive, we observed essentially no stimulation of CSF activity or mRNA when we used human umbilical vein EC (HUVEC) (Fig. 2*b*, Table 1). These results could be pertinent to the observation that different regions of the arterial wall show large variations in susceptibility to the

development of fatty streaks as well as advanced atherosclerotic lesions¹⁶.

Although most preparations of 'native' LDL failed to stimulate CSF expression, occasional preparations had some activity. We do not know whether this resulted from a low level of oxidized LDL *in vivo*¹⁷ or from slight oxidation during LDL

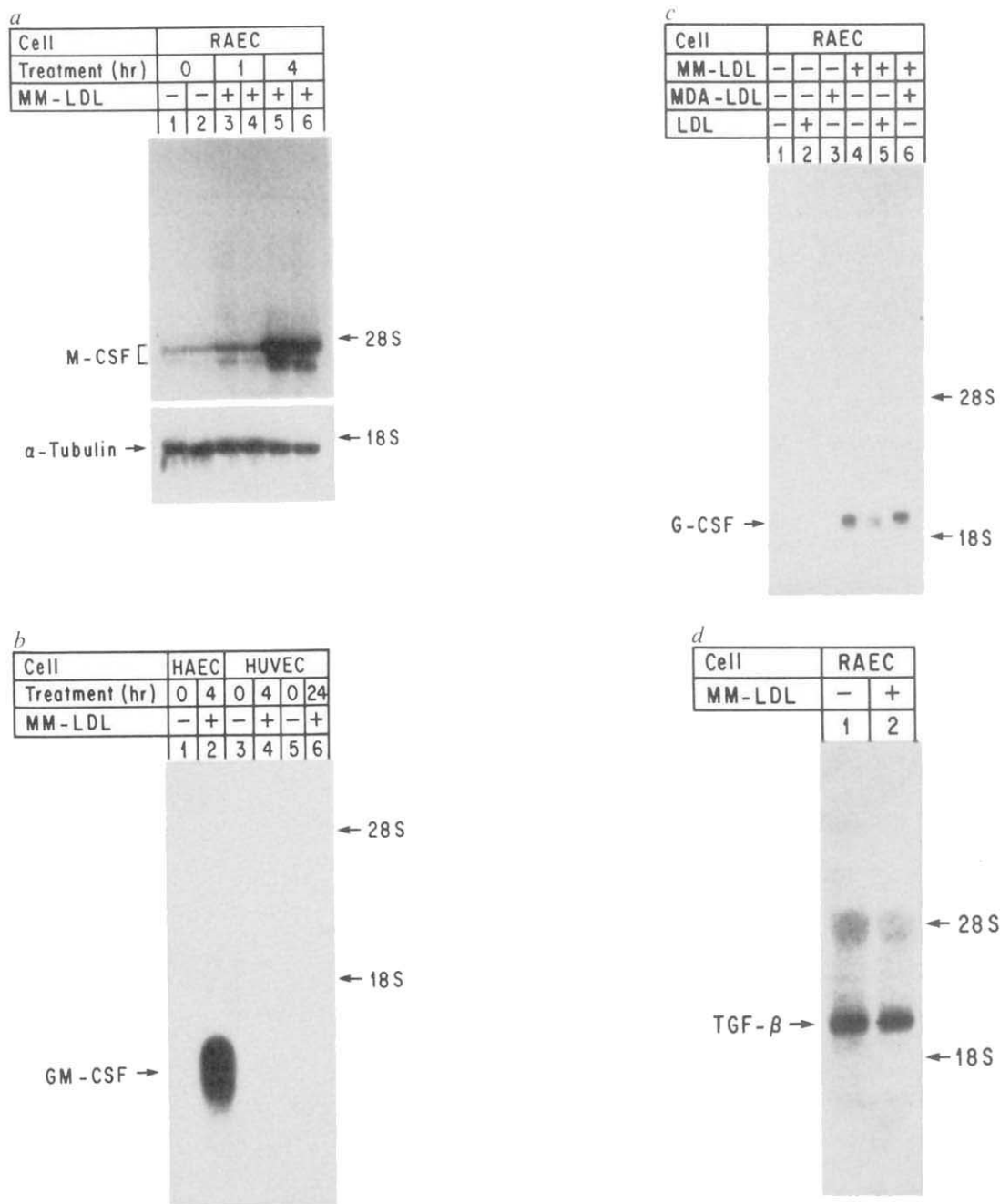


FIG. 2. Induction of CSF expression in aortic but not umbilical vein EC by treatment with MM-LDL. EC cultures were treated as described below with various human LDL preparations, and total RNA was extracted and examined for levels of CSF, α -tubulin and TGF- β mRNAs by northern blot analysis using human cDNA probes. *a*, Northern blot probed for M-CSF mRNA and α -tubulin mRNA. RNA was from duplicate cultures of RAEC treated with MM-LDL for 0 h (lanes 1,2), 1 h (lanes 3, 4) or 4 h (lanes 5, 6). *b*, Northern blot probed for GM-CSF mRNA. RNA was isolated from human aortic endothelial cells (HAEC) or human umbilical vein endothelial cells (HUVEC) treated with MM-LDL for 0 h (lanes 1, 3, 5), 4 h (lanes 2, 4) or 24 h (lane 6). *c*, Northern blot probed for G-CSF mRNA. RNA was from RAEC treated for 4 h with (lanes 4–6) or without (lanes 1–3) MM-LDL ($2 \mu\text{g ml}^{-1}$) in the presence of a 10-fold excess ($20 \mu\text{g ml}^{-1}$) native LDL (lanes 2 and 5) or

MDA-LDL (lanes 3 and 6). *d*, Northern blot probed for TGF- β mRNA. RNA was from RAEC cultured for 4 h in the absence (lane 1) or presence (lane 2) of MM-LDL. The migration positions of 28S and 18S ribosomal RNA are indicated.

METHODS. Primary cultures of RAEC (passages 6–17), HAEC (passages 7–10) and HUVEC (passages 1–10) were derived as previously described^{8,24,25}. Lipoproteins were added to endothelial cell cultures at a concentration of $2 \mu\text{g protein ml}^{-1}$ (RAEC) or $20 \mu\text{g ml}^{-1}$ (HAEC or HUVEC) in medium containing 10% FCS as previously described⁸. RNA isolation and northern blot analysis were performed as described in Fig. 1. The blots were probed and washed separately with isolated cDNA for human GM-CSF²⁸, M-CSF³¹, G-CSF³², TGF- β ³³ and α -tubulin³⁰ as in Fig. 1 except for G-CSF, which was washed in $1 \times \text{SSC}$, 0.1% SDS at 65°C .

TABLE 1 Effects of modified LDL on CSF mRNA expression in endothelial cells

mRNA*	Cell type	Treatment†	Relative mRNA Levels‡ (mean $\pm$ s.d.)	No. of Experiments	Fold induction
M-CSF	RAEC	Control	0.16 $\pm$ 0.03	6	1.0
		Native-LDL	0.15	1	0.9
		MDA-LDL	0.18	1	1.1
		MM-LDL (1 hr)	0.27	2	1.7
		MM-LDL (4 hr)	1.5 $\pm$ 0.3	6	9.4
		MM-LDL (24 hr)	0.91	2	5.7
	HAEC	Control	1.5	2	1.0
		Native-LDL	1.6 $\pm$ 0.3	3	1.0
		MM-LDL	3.2 $\pm$ 0.4	4	2.0
	HUVEC	Control	0.41	2	1.0
GM-CSF	RAEC	MM-LDL	0.53	2	1.3
		Control	0.9	2	1.0
		Native-LDL	1.1	2	1.2
		MDA-LDL	1.5	2	1.6
	HAEC	MM-LDL	5.6	2	6.1
		Control	0	2	ND§
		Native-LDL	0	3	ND
	HUVEC	MM-LDL	0.44 $\pm$ 0.05	4	>100
		Control	0	2	ND
		MM-LDL	0	2	ND
G-CSF	RAEC	Control	0	1	ND
		Native-LDL	0	1	ND
		MDA-LDL	0	1	ND
		MM-LDL	0.41	1	>100
TGF- β	RAEC	Control	0.37	2	1
		MM-LDL	0.37	2	1

* RNA isolated from endothelial cell cultures was examined for expression of mRNA for M-CSF, GM-CSF, G-CSF or TGF- β as described in Fig. 2.

† RAEC were treated with 2.0 $\mu\text{g ml}^{-1}$ native or modified LDL, whereas HAEC and HUVEC were treated with 20 $\mu\text{g ml}^{-1}$ native or modified LDL. A variety of MM-LDL preparations, obtained by either brief Fe^{2+} oxidation or prolonged storage at 4 °C, were used. The preparations contained ~5 nmol TBARS per mg cholesterol and most preparations contained undetectable levels of endotoxin (<5 pg per μg LDL cholesterol) as judged by the Limulus assay. Cells were collected 4 h after treatment, except where indicated.

‡ Northern blots were autoradiographed for 1–3 days and signals were quantitated by densitometric scanning. The levels of CSF mRNA in each experiment were normalized to α -tubulin mRNA levels and are expressed as relative levels.

§ ND, not detectable.

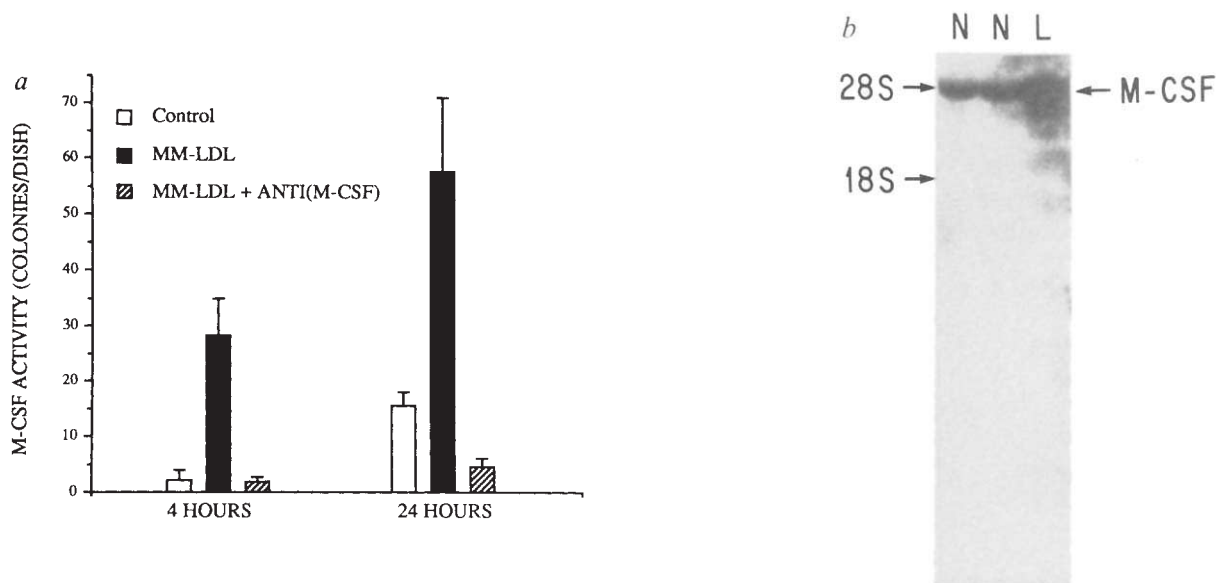


FIG. 3. Expression of M-CSF in aorta *in vitro* and *in vivo*. *a*, Induction of M-CSF activity in cultured HAEC. The cells were exposed to MM-LDL (20 μg per ml of protein) for either 4 h or 24 h and the resulting conditioned media were collected and tested for their ability to stimulate clonal proliferation of mouse bone marrow cells³⁴. Bone marrow cells (1×10^5 per dish) were plated in duplicate 35-mm Petri dishes in MEM containing 10% FCS, 0.3% agar and 0.1 ml test conditioned medium. Plates were incubated at 37 °C in 5% CO_2 and colonies containing ≥ 40 cells were counted on day 7. Mouse bone marrow cells are responsive to human M-CSF and G-CSF, but not to human GM-CSF. In some experiments, a neutralizing antibody, specific for human M-CSF was added to the conditioned medium. That

essentially all of the CSF activity (using mouse cells) can be neutralized with antibody to M-CSF, indicates that relatively little G-CSF is produced by the stimulated EC cells. MM-LDL added directly to bone marrow cells in soft agar did not stimulate colony formation. *b*, Northern blot of mRNA isolated from two normal rabbit aortas (N) and from a lesion (L) in the same region of the aorta in a rabbit maintained for 2 months on a high-cholesterol diet. Procedures for maintaining rabbits and assessing lesions were essentially as described¹⁸ and RNA isolation and northern blotting were as in Fig. 1. Similar inductions of M-CSF mRNA were observed using lesions from genetically hyperlipidemic (WHHL) rabbits or swine fed a high-fat diet (data not shown).

isolation. Previous studies have indicated that MM-LDL is taken up by the LDL receptor pathway⁸, and our preliminary results are consistent with this conclusion. An excess of 'native' LDL was therefore able to compete with MM-LDL for induction of G-CSF expression, whereas malondialdehyde-modified LDL (MDA-LDL), which is internalized by the scavenger receptor rather than the LDL receptor³, failed to compete (Fig. 2c).

It is not known whether induction of CSF expression in response to modified LDL occurs *in vivo*. But because oxidized LDL accumulates in atherosclerotic lesions^{3,4}, we examined the levels of CSF mRNA in lesions and in normal arteries of rabbits (Fig. 3b). The lesions were obtained from aortas of rabbits fed a high cholesterol diet and comparable regions of normal artery were obtained from matched rabbits fed a low-fat chow diet¹⁸. In three experiments, the levels of mRNA for M-CSF were elevated an average of about twofold, consistent with the possibility that induction of CSF expression by oxidized LDL occurs *in vivo*. Whether induction of GM-CSF or G-CSF occurs in lesions is unclear, because the levels of the mRNAs for these factors in whole-artery RNA were below the levels of detection by northern blotting. Alternatively, the levels of mRNA for IL-1- β , transforming growth factor- β (TGF- β) and the M-CSF receptor (*c-fms* gene product) were unchanged, whereas the levels of platelet-derived growth factor mRNA, which are elevated in human lesions¹⁹, increased about threefold (data not shown). Also supporting the possibility that oxidized LDL stimulates CSF expression *in vivo* is the finding that diet-induced hyperlipidemia in swine is associated with elevations of circulating monocytes and monocyte precursors in bone marrow²⁰.

Our studies indicate that mildly oxidized LDL is a specific and potent inducer of three separate CSFs in endothelial cells. Recently we have also observed that the induction of monocyte chemotactic protein occurs at the level of mRNA, but it is clear from those experiments that this protein is distinct from the CSFs (S. Cushing *et al.*, unpublished results). CSF expression could contribute to the initiation or progression of atherosclerotic lesions by affecting macrophage function and survival in the artery wall. For example, abnormal expression of GM-CSF in eye and muscle occurring in certain transgenic stocks is accompanied by massive macrophage accumulation and damage of these tissues²¹. Macrophages take up cholesterol to give rise to the foam cells that are a hallmark of the initial stages of atherosclerosis, and they also produce a host of growth factors that can affect processes such as smooth muscle cell proliferation²². The results described here indicate a possible link between lipid metabolism and growth-factor expression in the artery wall. □

28. Wong, G. G. *et al. Science* **228**, 810–815 (1984).
29. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6–13, (1983).
30. Salser, W. A., Chow, C.-H., Davis, R. C., Slovin, J. P. & Thomson, A. *Leukemia: Recent Advances in Biology and Treatment* (eds Gale, R. P. & Golde, D. W.) 349–379 (A. R. Liss, New York, 1985).
31. Wong, G. G. *et al. Science* **235**, 1504–1508 (1987).
32. Nagata, S. *et al. Nature* **324**, 73–77 (1986).
33. Derynck, R. *et al. Nature* **316**, 701–705 (1985).
34. Golde, D. W. & Cline, M. J. *J. clin. Invest.* **52**, 2981–2983 (1972).

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Interference with the assembly of a virus–host transcription complex by peptide competition

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INDUCTION of transcription of the immediate-early (IE) genes of herpes simplex virus (HSV) involves the assembly of a DNA-binding complex containing the cellular transcription factor Oct-1 and the virus regulatory protein Vmw65 (VP16)^{1–6}. Complex assembly can be observed using deletion variants of Vmw65 which lack the acidic C-terminal activation domain^{7–9} and are therefore defective for IE transactivation^{7,10}. Similar variants of Vmw65 interfere with IE activation by the normal protein¹⁰, and with HSV replication¹¹. It has therefore been suggested that dominant interfering products of viruses such as HSV and HIV could be used in a form of intracellular immunization against virus infection^{11–14}. Here we report that a small peptide overlapping a region of Vmw65 which is critical for complex assembly specifically inhibits assembly of the complex but has no observed effect on the DNA-binding activity of the cellular factor alone. Selective interference with the assembly of transcription complexes by short peptides corresponding to functionally critical regions of virus regulatory proteins may be more feasible than the use of defective polypeptides as an antiviral strategy based on competitive interference.

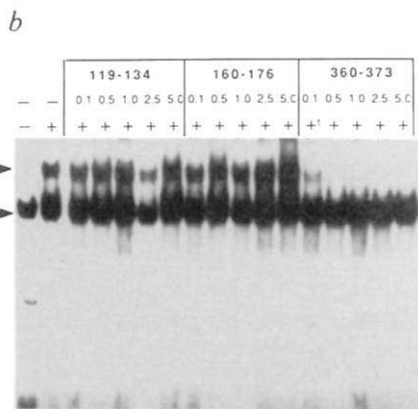
To identify the sequences within Vmw65 required for complex formation, a series of N- and C-terminal truncated proteins were produced by overexpression in tissue culture cells. More refined variants with single amino-acid substitutions were also used and the region encompassing residues 360–386 was shown to be crucial for complex formation with Oct-1 (TRF). Our results (R.G. and P.O'H., manuscript submitted)⁷ show that the truncated mutant containing the N-terminal 388 residues (RG39) formed a complex (TRF.C) as efficiently as the full-length protein, whereas deletion of the four amino acids from residues 388 to 385 (RG46) almost completely abolished complex formation (Fig. 1a). Mutants with more extensive deletions (RG43, RG40) did not form any detectable complex.

A search for homologies to Vmw65 has so far failed to identify any proteins with significant global alignments other than ORF10 of varicella zoster virus, but we have observed a strong local homology to the protein p3 of the bacteriophage ϕ 29 (Fig. 1a). This phage protein is involved in a terminal protein-mediated DNA replication initiation process^{15,16} potentially similar to that involving Oct-1 in adenovirus^{17–19}. Furthermore, as the area of local homology to Vmw65 encompasses a crucial boundary for Vmw65/Oct-1 complex formation, in a region where single amino-acid substitutions completely abolish complex assembly (R.G. and P.O'H., manuscript submitted), we propose that this region of Vmw65 is vital for complex formation, potentially as a site of direct protein–protein or protein–DNA interactions.

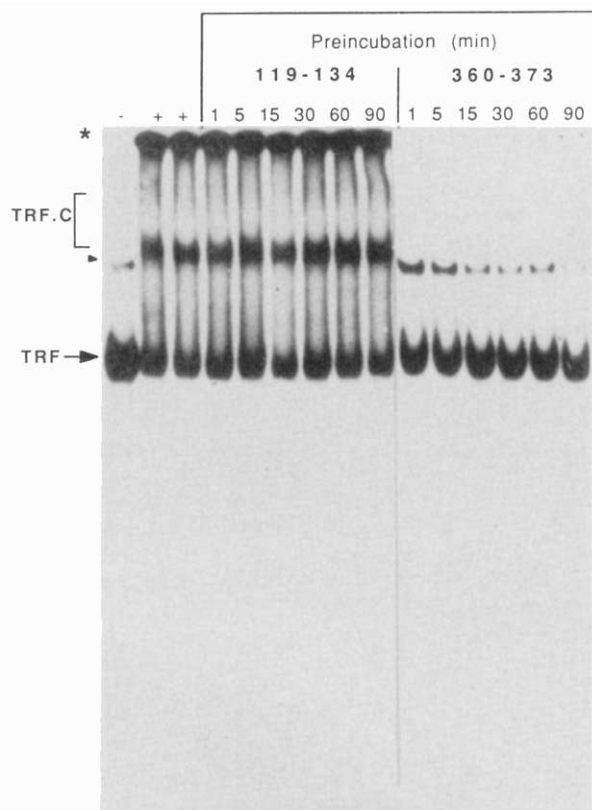
To provide additional evidence for a critical requirement for this region, we performed a series of experiments in which a

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1. Morton, R. E., West, G. A. & Hoff, H. F. *J. Lipid Res.* **27**, 1124–1134 (1986).
2. Hoff, H. F. & Gaubatz, J. W. *Arteriosclerosis* **42**, 273–297 (1982).
3. Haberland, M. E., Fong, D. & Cheng, L. *Science* **241**, 215–218 (1988).
4. Palinski, W. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 1372–1380 (1988).
5. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witztum, J. L. *New Engl. J. Med.* **320**, 915–924 (1989).
6. Kita, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 5928–5931 (1987).
7. Carew, T. E., Shwenke, D. C. & Steinberg, D. *Proc. natn. Acad. Sci. U.S.A.* **87**, 7725–7729 (1987).
8. Berliner, J. A. *et al. J. clin. Invest.* (in the press).
9. Berliner, J. A. *et al. Arteriosclerosis* **6**, 254–258 (1986).
10. Metcalf, D. *Nature* **339**, 27–30 (1989).
11. Wang, J. M., Griffin, J. D., Rambaldi, A., Chen, Z. G. & Mantovani, A. *J. Immun.* **141**, 575–579 (1988).
12. Bussolino, F. *et al. Nature* **337**, 471–473 (1989).
13. Navab, M. *et al. J. clin. Invest.* **82**, 1853–1863 (1988).
14. Clark, S. C. & Kamen, R. *Science* **236**, 1229–1237 (1987).
15. Libby, P. *et al. Am. J. Path.* **124**, 179–185 (1986).
16. Gerrity, R. G., Naito, H. K., Richardson, M. & Schwartz, C. J. *Am. J. Path.* **95**, 775–785 (1984).
17. Avogaro, P., Bon, G. B. & Cazzalato, G. *Arteriosclerosis* **8**, 79–87 (1988).
18. Tsukada, T., Rosenfeld, M., Ross, R. & Gown, A. M. *Arteriosclerosis* **6**, 601–613 (1986).
19. Barrett, T. B. & Benditt, E. P. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1099–1103 (1987).
20. Averill, L. E., Meagler, R. C. & Gerrity, R. G. *Am. J. Path.* **135**, 369–377 (1989).
21. Lang, R. A. *et al. Cell* **51**, 675–686, (1987).
22. Ross, R. *New Engl. J. Med.* **314**, 488–500 (1986).
23. Kosugi, K., Morel, D. W., DiCorleto, P. E. & Chisholm, G. M. *J. Cell Physiol.* **130**, 311–320 (1987).
24. Gimbrone, M. A., Cotran, R. S. & Folkman, J. *J. Cell Biol.* **60**, 673–684, (1974).
25. Baker, D. P. *et al. Arteriosclerosis* **4**, 248–255, (1984).
26. Chomzynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156–159 (1987).
27. Rajavashisth, T. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 1157–1161 (1987).



METHODS. Aliquots of a HeLa cell nuclear extract (1.0 μg) were incubated on ice for 30 min without or with various doses of each of the peptides in the presence of 0.5 μg of poly(dI-dC). Vmw65, purified by ion-exchange chromatography from infected cell extracts, was then added and the samples incubated at 25 $^{\circ}\text{C}$ for 20 min. The radiolabelled probe (TAAT24, CCATGAGATCTCGTGCATGCTAATGATATCTT) encompassing residues -171 to -149 of the IE 110K (where 110K is the M_r of the protein) gene of HSV-1 was then added and incubation continued for a further 20 min. DNA-binding complexes were separated on 4% polyacrylamide gels (acrylamide: bis-acrylamide, 39:1) at 200 V for 2.5 h and the complexes located by autoradiography of the dried gels.



METHODS. Aliquots of HeLa cell nuclear extract (1.0 μ g) were preincubated with 5 μ g peptide 119-134 or peptide 360-373 for the times indicated. After preincubation, Vmw65 was added to each sample and incubation continued at 25 °C for 20 min. DNA-binding complexes were separated on a 4% non-denaturing gel after a further 20 min incubation with the TAAT24 probe.

peptide (residues 360–373), encompassing the region of homology, was used in an attempt to 'compete out' formation of TRF.C by normal full-length Vmw65. Peptide 360–373 or control peptides (Fig. 1a) were added in increasing amounts to a nuclear extract before the addition of Vmw65, and the effect on TRF and TRF.C formation were examined by gel retardation analysis.

Typical results of these experiments are shown in Fig. 1b. The two lanes to the left show TRF in the nuclear extract and the formation of TRF.C on addition of Vmw65. The next lanes show the effect of different doses of each of the peptides on the binding activities of TRF and TRF.C, as indicated. Neither the 119–134 or the 160–176 peptides had any significant effect on TRF or the formation of TRF.C at any of the doses tested. In contrast, incubation with the peptide 360–373 substantially inhibited TRF.C formation at the lowest dose tested and virtually abolished it as doses of 1.0 μg or higher. Importantly, in the same assay, this peptide had no effect on independent TRF-binding activity at any dose tested.

We then examined the effect of preincubation time on inhibitory activity and whether complex formation was reversible if the peptide was added after Vmw65 instead of before. The results (Fig. 2) indicate that the inhibitory effect of the peptide was extremely rapid, requiring only 1–5 min preincubation with the HeLa-cell extract to prevent TRF.C formation (Fig. 2, peptide 360–373), with no further decrease in complex formation on longer preincubation times. As expected, preincubation with the control peptide had no effect on TRF.C formation, irrespective of the time of preincubation (Fig. 2, peptide 119–134). Furthermore, no effect was detected on the appearance of TRF.C complex if peptide 360–373 was added after HeLa and Vmw65 incubation, or after formation of the complete DNA-binding

complex by addition of the radiolabelled TAATGARAT probe (Fig. 3). As shown (Fig. 3, protocol 1), preincubation of the peptide with the HeLa extract was necessary to prevent the appearance of TRF.C formation in the presence of Vmw65. These results indicate that Vmw65 interacts with a cellular component in the absence of target DNA sequences, and that it is this step that is the target of peptide inhibition. That the peptide had no effect on preformed TRF.C complex or on independent TRF binding indicates that it is highly unlikely that peptide inhibition by preincubation was due to some non-specific artefactual effect.

The simplest interpretation of these data is that the peptide 360–373 inhibits TRF.C formation by competitive interference, presumably by direct interaction with the cellular components of the complex.

Residues within the homoeodomain of Oct-1 have been shown to be important for assembly of the complex with Vmw65²⁰ and could represent sites of direct protein–protein contact. However, we do not conclude from our results that this region of Oct-1, or even Oct-1 itself, is necessarily the target for the peptide, as inhibition could be mediated by interaction with another component of the complex.

As complex formation correlates with IE activation^{3,7,21}, it is very likely that the peptide would prevent IE induction by Vmw65. This proposal will have to be tested by peptide micro-injection, for example, and subsequent virus infection or Vmw65 transfection. Moreover, because Vmw65 transactivation of IE expression is also required for normal virus replication and for virulence in mice²², peptide interference of Vmw65 function may present a feasible route for inhibition of HSV replication.

Previous studies have demonstrated the inhibition of certain virus-encoded enzymes, for example the HSV ribonucleotide reductase^{23,24} and the HIV-1 protease²⁵, by peptide competition. In the case of the HSV ribonucleotide reductase, the inhibitory peptide directly associates with one of the enzyme subunits, preventing interaction with the other subunit²⁶. Our results indicate that following the identification of a functionally critical region in a virus regulatory protein, peptide competition may also be used for the inhibition of transcription complex assemblies. The simian virus 40 T antigen–retinoblastoma protein interaction can also be competed out by a peptide encompassing a required domain of the large T antigen²⁷. Peptide interference may, therefore, be extended to the selective modulation or prevention of virus-induced or host-cell gene expression in other contexts. □

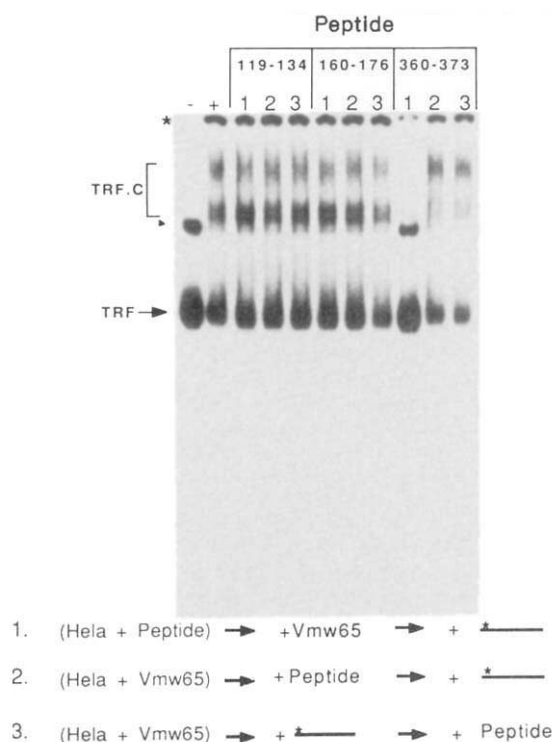


FIG. 3 Inhibition of TRF.C assembly. Peptide 360–373 must be incubated with the HeLa cell extract before Vmw65 is added. To determine the step at which the peptide 360–373 affects TRF.C assembly, HeLa cell extract (1 μg), Vmw65, peptide (5 μg) and TAATGARAT probe (*—) were added in various orders, 1, 2, 3. Each incubation step was for 20 min at room temperature. Complexes (labelled as for Fig. 2) were separated on 4% non-denaturing gels.

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- O'Hare, P. & Goding, C. R. *Cell* **52**, 435–445 (1988).
- Preston, C. M., Frame, M. C. & Campbell, M. E. *Cell* **52**, 425–434 (1988).
- O'Hare, P., Goding, C. R. & Haigh, A. *EMBO J.* **7**, 4231–4238 (1988).
- McKnight, J. L., Kristie, T. M. & Roizman, B. *Proc. natn Acad. Sci. U.S.A.* **84**, 7061–7065 (1987).
- Gerster, T. & Roeder, R. G. *Proc. natn Acad. Sci. U.S.A.* **85**, 6347–6351 (1988).
- apRhyas C. M., Clufo, D. M., O'Neill, E. A., Kelly, T. J. & Hayward, G. S. *J. Virol.* **63**, 2798–2812 (1989).
- Greaves, R. P. & O'Hare, P. *J. Virol.* **63**, 1641–1650 (1989).
- Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. *Nature* **335**, 563–564 (1988).
- Cousens, D. J., Greaves, R., Goding, C. R., & O'Hare, P. *EMBO J.* **8**, 2337–2342 (1989).
- Triezenberg, S. J., Kingsbury, R. C. & McKnight, S. L. *Genes Dev.* **2**, 718–729 (1988).
- Friedman, A. D., Triezenberg, S. J. & McKnight, S. L. *Nature* **335**, 452–454 (1988).
- Baltimore, D. *Nature* **335**, 395–396 (1988).
- Green, M., Ishino, M. & Lowenstein, P. M. *Cell* **58**, 215–223 (1985).
- Rinsky, L., Duc Dodon, M., Dixon, E. P. & Green, W. C. *Nature* **341**, 453–456 (1989).
- Herrera, L., Salas, M. & Carrascosa, J. L. *Virology* **155**, 289–292 (1986).
- Zaballos, A., Mellado, R. P. & Salas, M. *Gene* **63**, 113–121 (1988).
- Prujin, G. J., van Driel, W. & van der Vliet, P. C. *Nature* **322**, 656–659 (1986).
- Prujin, G. J., van Driel, W., van Miltenburg, R. T. & van der Vliet, P. C. *EMBO J.* **6**, 3771–3778 (1987).
- Prujin, J. M., van der Vliet, P. C., Dathan, N. A. & Mattaj, J. W. *Nucleic Acids Res.* **17**, 1845–1863 (1989).
- Stern, S., Tanaka, M. & Herr, W. *Nature* **341**, 642–650 (1989).
- Ace, C. I., Dalrymple, M. A., Ramsay, F. M., Preston, V. G. & Preston, C. M. *J. gen. Virol.* **69**, 2595–2605 (1988).
- Ace, C. I., MacKee, T., Ryan, M., Cameron, J. M. & Preston, C. M. *J. Virol.* **63**, 2260–2269 (1989).
- Cohen, E. A., Gaudreau, P., Brazeau, P. & Langelier, Y. *Nature* **321**, 441–443 (1986).
- Dutia, B. M., Frame, M. C., Subka-Sharpe, J. H., Clark, W. N. & Marsden, H. S. *Nature* **321**, 439–441 (1986).
- Meek, T. et al. *Nature* **343**, 90–92 (1990).
- Paradis, H., Gaudreau, P., Brazeau, P. & Langelier, Y. *J. biol. Chem.* **263**, 16045–16050 (1988).
- DeCaprio, J. A. et al. *Cell* **58**, 1085–1095 (1989).

Functional heterogeneity of mammalian TATA-box sequences revealed by interaction with a cell-specific enhancer

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A REGULATORY element upstream of the human myoglobin gene functions as a muscle-specific enhancer (MSE) in conjunction with core promoter elements of the myoglobin gene, but not in combination with the simian virus 40 (SV40) early promoter¹. These two promoters differ in the sequences of their 'TATA boxes': for the myoglobin gene, the sequence is TATAAAA (ref. 2), whereas for SV40, the sequence is TATTTAT (ref. 3). We have now tested the hypothesis that this sequence difference is responsible for the differential response of the promoters to the MSE. We found that when the TATA box sequence of the myoglobin promoter was changed to that of the SV40 promoter, responsiveness to the MSE was abolished; conversely, when the SV40 TATA box sequence was changed to that of the myoglobin promoter, the promoter became responsive to the MSE. We conclude that mammalian TATA-box elements are functionally heterogeneous, and suggest

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Myoglobin promoter SV40 early promoter

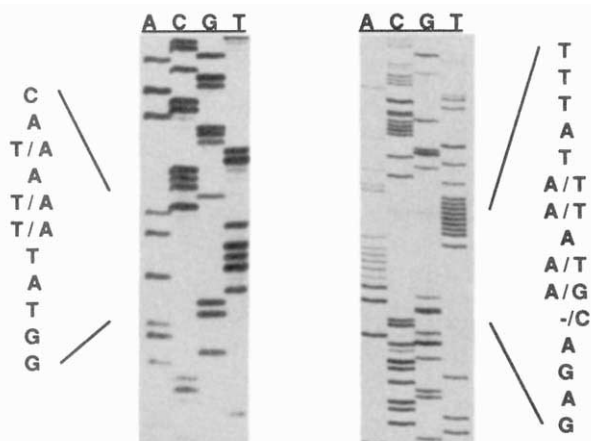


FIG. 1 Nucleotide sequence of mutant TATA-box element in the human myoglobin gene promoter and the SV40 early promoter linked to the bacterial CAT gene in plasmid constructs. Mutated bases are indicated to the left of the slash, with the native sequence on the right. Because of the use of different sequencing primers, the 5'-to-3' direction on the coding strand is shown upwards for the myoglobin promoter and downwards for the SV40 early promoter.

METHODS. Upstream sequences from the human myoglobin gene² were inserted into the reporter vector pCAT20 as previously described¹. SV40 early promoter sequences lacking enhancers linked to the CAT gene were derived from plasmid pA10CAT2 (ref. 3). Restriction fragments containing these native promoter sequences were subcloned into a phagemid vector (pBST; Stratagene) to generate single-stranded DNA for oligonucleotide-directed mutagenesis of the TATA-box elements using the phosphothiorate procedure¹⁴. The nucleotide sequence of individual clones was determined by dideoxynucleotide sequencing from double-stranded template, and mutants selected for transfection experiments.

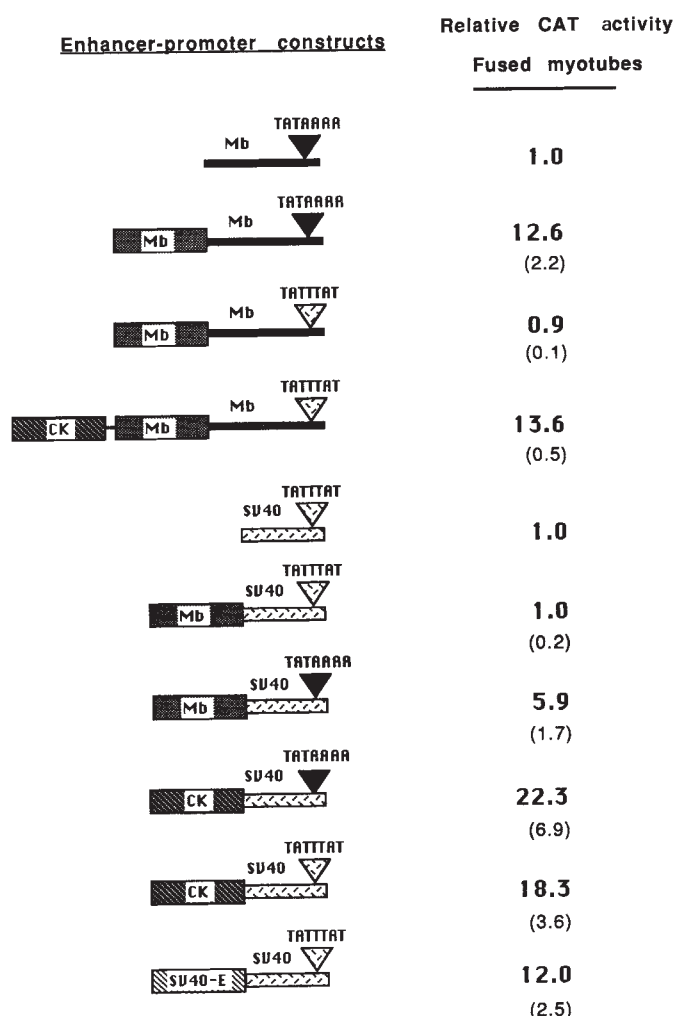


FIG. 2 Relative expression of CAT in skeletal myotubes from constructs containing native and mutant TATA-box elements in the human myoglobin (Mb) gene promoter and SV40 early promoter. Each construct is defined in terms of three variables: (1) core promoter regions from the human myoglobin gene promoter or SV40 early promoter; (2) the presence or absence of enhancers (shaded boxes) from the human myoglobin gene, the mouse M-creatine kinase (CK) gene, or the SV40 72-bp repeat region (SV40-E); and (3) TATA elements TATAAAA (native form in the human myoglobin gene promoter) or TATTTAT (native form in the SV40 early promoter). Mean values of CAT activity (multiples of background activity expressed from constructs lacking enhancers) from four to eight transfection experiments for each construct are shown in bold type and s.e.m. is shown in parentheses.

METHODS. Cultures of myoblasts and fibroblasts were established from 12-day-old chick embryos and transfected by calcium phosphate co-precipitation, or in later experiments by liposome fusion (Lipofectin, Bethesda Research Laboratories), and whole-cell extracts analysed for CAT activity 6 days after transfection as described previously¹. Fusion of cells into multinucleated myotubes was apparent in all myocyte cultures by 4 days after transfection. Plasmids were isolated from at least three separate large-scale preparations by alkaline lysis, and were purified by column chromatography (5'-3'), precipitation with PEG 8000, and repeated ethanol washes. CAT activity in each extract was measured as an initial rate (linear over several hours) as assessed by diffusion of [¹⁴C] acetylchloramphenicol from the aqueous phase into liquid scintillation fluor. To confirm that low-level expression of certain constructs was not an artefact of low transfection efficiency, some cultures were co-transfected with 2 µg of another construct, in which the human placental alkaline phosphatase gene is driven constitutively by the long terminal repeat of the Rous sarcoma virus. High-level activity of this latter reporter gene product was evident in cultures co-transfected with inactive CAT constructs (data not shown).

that this heterogeneity reflects differential interactions with distinctive TATA box-binding factors, only some of which can act cooperatively with MSE-binding proteins to generate an active transcriptional complex.

To test the hypothesis that the variation in TATA-box sequence is responsible for the differences in enhancing activity of the myoglobin upstream sequences, we constructed a series of mutant promoters linked to the chloramphenicol acetyltransferase (CAT) gene that are distinguished by permutations of the following variables: (1) the presence or absence of an enhancer (either the myoglobin gene MSE, a different muscle-specific enhancer from the mouse M-creatine kinase (M-CK) gene^{4,5}, or the SV40 72-base pair (bp) repeat region³); (2) the core promoter (exclusive of the TATA box) of either the myoglobin gene promoter or the SV40 early promoter; and (3) the TATA-box element (TATAAAA or TATTTAT). We verified the identity of each construct by DNA sequencing (Fig. 1), and analysed promoter activity in transient expression assays in primary cultures of fibroblasts or skeletal myotubes from chick embryos.

As observed in previous experiments¹, the myoglobin enhancer increased expression from myoglobin core promoter sequences by about 10-fold in skeletal myotubes (Fig. 2). But, mutation of the myoglobin TATA-box sequence (TATAAAA) so that it was identical to that of the SV40 early promoter (TATTTAT) abolished MSE activity of the myoglobin upstream region, even though the remaining myoglobin core promoter sequences (nucleotide positions -204 to +7) were unaltered. RNase protection assays (Fig. 3) indicated that the abundance of the messenger RNA correlated with CAT activity.

SV40 promoter constructs that lack enhancers expressed little CAT activity in myotubes, irrespective of the TATA-box sequence. In addition, we confirmed previous findings¹ that the MSE region had no enhancing activity when linked to the SV40 early promoter with a native TATA-box sequence (TATTTAT). But, mutation of the SV40 TATA-box element to form the TATAAAA sequence found in the human myoglobin gene unmasked MSE activity of the myoglobin upstream region (Fig. 2). As assessed by RNase protection assays (Fig. 3), the principal sites of transcriptional initiation from the SV40 early promoter with a mutant TATA-box element linked to the MSE were identical to those of transcriptional initiation from the native SV40 early promoter linked to the SV40 enhancer. The myoglobin upstream region failed to enhance transcription above background levels in fibroblasts, irrespective of either the core promoter to which it was linked, or the TATA-box sequence (CAT activity <5% that of pSV2CAT, $n = 4$).

In contrast to the effects of TATA-box mutations on expression of constructs driven by the myoglobin MSE, the muscle-specific upstream enhancer from the mouse M-CK gene directed high expression of CAT activity from the SV40 early promoter (Fig. 2), and transcripts were correctly initiated (Fig. 3), irrespective of which TATA-box sequence was included in the construct. Therefore, selective synergism between the myoglobin MSE and certain TATA-box elements is not a universal feature of muscle-specific enhancers.

We conclude that the sequences TATAAAA and TATTTAT are functionally distinct in terms of their ability to serve as core promoter elements in conjunction with the myoglobin gene upstream enhancer. Heterogeneity of eukaryotic TATA-box elements was first observed in the *his3* gene of *Saccharomyces cerevisiae* by Struhl⁶⁻⁸, who noted that certain TATA-box sequences are functional during low-level constitutive expression, but fail to support inducible expression mediated by upstream activator sequences during amino-acid starvation. In mammalian cells, Simon *et al.*⁹ identified mutations of the TATAAAA sequence found in the human Hsp70 promoter (including conversion to TATTTAT) that result in a loss of responsiveness to *trans*-activation by adenovirus E1A, but preserve heat inducibility.

The results presented here are evidence of a restricted interaction between a cell-specific enhancer and discrete TATA-box sequences. A model that illustrates a possible mechanism of this functional diversity of TATA-box sequences is shown in Fig. 4. We suggest that mammalian cells express a family of TATA-box binding proteins that differ in their relative affinities for specific

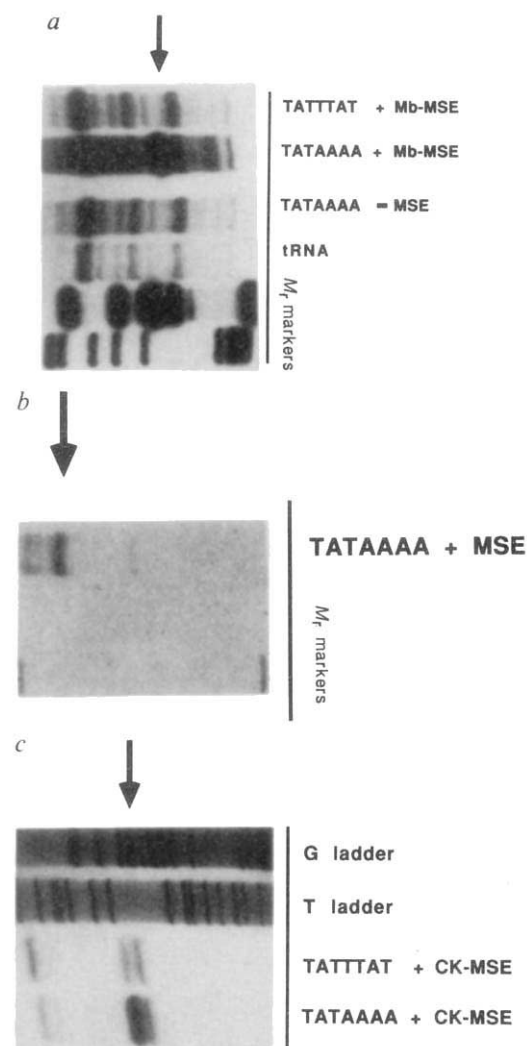
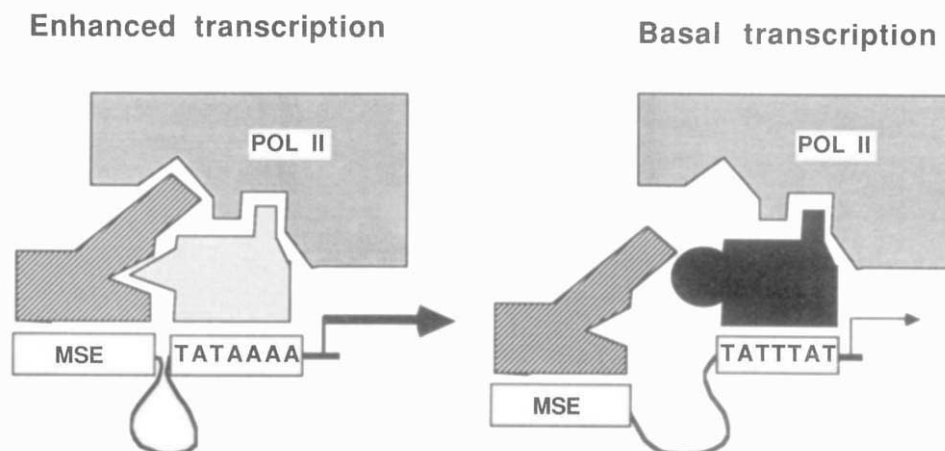


FIG. 3 Transcriptional start-site analyses of CAT mRNA in cells transfected with constructs driven by the myoglobin promoter (a) or the SV40 early promoter (b, c). a, Correctly initiated transcripts (arrows) were evident in myotubes transfected with constructs containing TATAAAA sequences linked to the myoglobin (Mb) upstream enhancer (+MSE), but were not detectable in the absence of an enhancer (-MSE), or when the TATA-box element was mutated (TATTTAT). b, When linked to the myoglobin MSE, correctly initiated transcripts were observed only after the TATTTAT sequence native to the SV40 early promoter was mutated to the TATAAAA sequence. c, By contrast, constructs driven by the M-creatine kinase (CK) MSE expressed correctly initiated transcripts with either TATA-box element. Relative molecular mass (μ), markers, sequence ladders, and nonspecific probe fragments observed after annealing to *E. coli* transfer RNA are also shown.

METHODS. RNase protection assays (a, b) were performed with total RNA after annealing to [³²P]-labelled antisense transcripts generated *in vitro* with T7 polymerase from either a myoglobin promoter/CAT fusion fragment (a) or an SV40/CAT fusion fragment (b) subcloned into phagemid pBST (Stratagene). Primer extension assays with reverse transcriptase were used to assess start sites of constructs containing the M-CK enhancer (c). The primer was a synthetic oligonucleotide (20mer) corresponding to a sequence in the protein coding region of the CAT gene. For determination of the termination point of extension products, the same primer was used in dideoxynucleotide sequencing reactions with the SV40/CAT construct as template.

FIG. 4 Schematic model of possible mechanism for functional heterogeneity of the TATAAAA and TATTTAT elements based on differential binding of discrete cognate binding factors that differ in their ability to interact with factors binding the myoglobin MSE. The TATAAAA binding factor includes a domain that forms a complex with MSE-binding factors, resulting in enhanced transcription. By contrast, the TATTTAT-binding factor forms this higher-order complex less efficiently, and only basal levels of transcription are observed. Alternatively, an identical TATA box-binding factor could assume different conformations when bound to TATAAAA versus TATTTAT, only one of which permits effective interaction with the enhancer. Pol II, RNA polymerase II.



TATA-box sequences. Although all proteins of this class are capable of interaction with RNA polymerase II and other general components of the pre-initiation complex, we hypothesize that these proteins differ in their ability to form higher-order complexes through protein-protein interactions with enhancer-binding proteins. An alternative model is that, when bound to varying TATA-box sequences, a single form of TATA-box binding protein can assume different configurations, only a subset of which can be recognized by certain enhancer-binding proteins.

These models must ultimately be tested with purified components in cell-free systems. Roeder and colleagues¹⁰⁻¹³ have purified a eukaryotic TATA-box binding factor (TFIID) and studied its interaction with various viral and mammalian promoters. Single-base mutations of TATA-box sequences can alter the affinity for binding of TFIID (ref. 12), with concomitant changes in transcription. In addition, binding of other factors to upstream regulatory sites can result in large changes in the specific nucleotides protected from DNase I by preparations of TFIID¹²⁻¹³. These studies support the concept of protein-protein interactions between TATA-box-binding proteins and other transcription factors, and indicate approaches by which the interactions of proteins binding to the myoglobin upstream

enhancer and specific TATA-box binding factors may be explored. □

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- Devlin, B. H., Wefald, F. C., Kraus, W. E., Bernard, T. S. & Williams, R. S. *J. biol. Chem.* **264**, 13896-13901 (1989).
- Weller, P., Jeffreys, A. J., Wilson, V. & Blanchetot, A. *EMBO J.* **3**, 439-446 (1984).
- Laird, L. A., Khoury, G., Gorman, C., Howard, B. & Gruss, P. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6453-6457 (1982).
- Sternberg, E. A. *et al. Molec. cell. Biol.* **8**, 2896-2909 (1988).
- Jaynes, J. B., Johnson, J. E., Buskin, J. N., Gartside, C. L. & Hauschka, S. D. *Molec. cell. Biol.* **8**, 62-70 (1988).
- Struhl, K. *Molec. cell. Biol.* **6**, 3847-3853 (1986).
- Chen, W. & Struhl, K. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2691-2695 (1988).
- Harbury, P. A. B. & Struhl, K. *Molec. cell. Biol.* **9**, 5298-5304 (1989).
- Simon, M. C., Fisch, T. M., Benecke, B. J., Nevins, J. R. & Heintz, N. *Cell* **52**, 723-729 (1988).
- Sawadogo, M. & Roeder, R. G. *Cell* **43**, 165-175 (1985).
- Huang, D.-H., Horikoshi, M. & Roeder, R. G. *J. biol. Chem.* **263**, 12596-12601 (1988).
- Horikoshi, M., Carey, M. F., Kakidani, H. & Roeder, R. G. *Cell* **54**, 655-669 (1988).
- Horikoshi, M., Hai, T., Lin, Y.-S., Green, M. R. & Roeder, R. G. *Cell* **54**, 1033-1042 (1988).
- Nakamaye, K. I. & Eckstein, F. *Nucleic Acids Res.* **14**, 9679-9698 (1986).

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Evolutionary transfer of the chloroplast *tufA* gene to the nucleus

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EVOLUTIONARY gene transfer is a basic corollary of the now widely accepted endosymbiotic theory, which proposes that mitochondria and chloroplasts originated from once free-living eubacteria¹. The small organellar chromosomes are remnants of larger bacterial genomes, with most endosymbiont genes having been either transferred to the nucleus² soon after endosymbiosis^{3,4} or lost entirely, with some being functionally replaced by pre-existing nuclear genes^{5,6}. Several lines of evidence indicate that relocation of some organelle genes could have been more recent. These include the abundance of non-functional organelle sequences of recent origin in nuclear DNA^{7,8}, successful artificial transfer of functional organelle genes to the nucleus⁹, and several examples

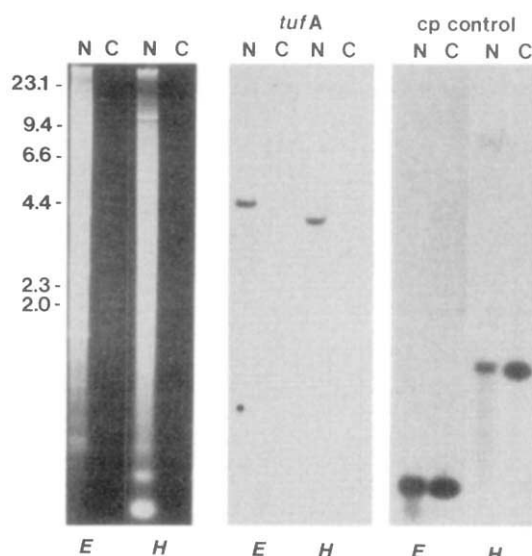
of recently lost organelle genes^{4,10}, although none of these is known to have been replaced by a nuclear homologue that is clearly of organellar ancestry. We present gene sequence and molecular phylogenetic evidence for the transfer of the chloroplast *tufA* gene to the nucleus in the green algal ancestor of land plants.

The *tufA* gene, encoding chloroplast protein synthesis elongation factor Tu (EF-Tu), was first identified as a chloroplast gene in the green alga *Chlamydomonas reinhardtii* by filter hybridization with an *Escherichia coli* gene probe¹¹ and in *Euglena gracilis* by complete sequencing of the chloroplast gene¹². Our attempts to hybridize the putative *Chlamydomonas tufA* to various land plant chloroplast DNAs were unsuccessful, and complete sequencing of three land plant chloroplast chromosomes^{10,13} has confirmed the absence of *tufA* from a nonvascular (*Marchantia polymorpha*) and two flowering (rice and tobacco) land plants.

We looked for *tufA* in land plant nuclear DNA by Southern blot analysis of *Arabidopsis thaliana* DNA. Figure 1 shows that the putative *Chlamydomonas tufA* hybridizes to a single fragment in *Arabidopsis* nuclear DNA digested with either *EcoRI* or *HindIII*. There is no hybridization detected with *Arabidopsis* chloroplast DNA (Fig. 1) or mitochondrial DNA (data not shown), despite overloading of these sequences relative to single-copy nuclear sequences (Fig. 1, cp control). Thus, *tufA* appears to be present in *Arabidopsis* in nuclear DNA exclusively.

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FIG. 1 A *Chlamydomonas* chloroplast *tufA* probe hybridizes to *Arabidopsis* nuclear DNA. Nuclear (N) and chloroplast (C) DNAs from *Arabidopsis thaliana* were digested separately with the restriction enzymes *Eco*RI (E) and *Hind*III (H) and fractionated on a 0.9% agarose gel (left panel). A nylon filter replica of the gel shown was hybridized sequentially with a *Chlamydomonas tufA* gene-specific probe (400 bp *Pst*I-*Eco*RI) and a *Brassica campestris* chloroplast DNA probe (a 600-bp *Eco*RI fragment of a 9.0-kb *Sac*I clone). The latter shows overloading of the chloroplast lane relative to chloroplast contamination in the nuclear DNA. Sizes in kb are indicated on the left. METHODS. 5' and 3' fragments of the *Chlamydomonas tufA* gene¹¹ (1.7 kb *Bam*HI-*Eco*RI and 1.3 kb *Eco*RI fragments, respectively) were subcloned from a chloroplast DNA library (gift from J. Boynton and N. Gillham). A 400-bp *Pst*I-*Eco*RI fragment, shown to be internal to the gene by hybridization with *Euglena tufA*¹² (gift from R. Helling), was subcloned from the *Bam*HI-*Eco*RI fragment. Nuclear DNA was extracted from Percoll-gradient-purified nuclei of *Arabidopsis thaliana*, strain Columbia (gift from C. Somerville) as described²⁴, omitting pretreatment of leaves with ether. Crude leaf extract was concentrated at 1,000g and layered over 30%/60% Percoll step-gradients. Nuclei were collected from the 30%/60% interface and then pelleted through 45% Percoll. Chloroplast DNA was extracted from sucrose gradient-purified chloroplasts²⁵. Restriction enzyme digestion, agarose gel electrophoresis, Southern transfer (without liquid reservoir), preparation of ³²P-labelled probes, and hybridization were as described²⁶. Nylon filters (Zetabind; AMF Cuno Inc.) were prepared as recommended by the manufacturer, apart from baking at 80 °C under vacuum for one hour. Hybridizations were at 60 °C, and after hybridization filters were washed once at room temperature and three times at 60 °C in 2×SSC, 0.5% SDS. Probes for



hybridization were fractionated in 1.0% low-gelling-temperature agarose and nick-translated directly in agarose slices.

The *Arabidopsis tufA*-hybridizing fragment was isolated from a genomic DNA library and sequenced together with the *Chlamydomonas tufA*. Both loci contain a single, uninterrupted open reading frame of 476 (*Arabidopsis*) and 418 (*Chlamydomonas*) codons (Fig. 2). There are an extra 201 nucleotides at the 5' end of the *Arabidopsis* open reading frame which are absent in all other known eubacterial and chloroplast *tufAs*. This sequence seems to encode a typical chloroplast transit peptide, which functions in subcellular targeting of nuclear-encoded chloroplast proteins¹⁴, in that it is rich in serine and threonine, lacks acidic residues and has a net positive charge. The rest of the *Arabidopsis* sequence aligns throughout with the entire *Chlamydomonas* sequence, except for a 27-nucleotide insertion (nucleotides 1080–1106) which, by comparison with other EF-Tus^{12,15,16}, is unique to *Chlamydomonas*. Overall sequence similarity between the two genes is 77% for the amino acids and 67% for nucleotides. No similarity is apparent between the putative transit peptide sequence of *Arabidopsis* and the 5' flanking DNA of the *Chlamydomonas tufA*.

We used northern blotting to show that the *Arabidopsis tufA* gene is actively expressed. A single *tufA* transcript of ~2.0 kilobases (kb) is enriched in poly(A)⁺ RNA and depleted in poly(A)⁻ RNA, a pattern similar to that found for *cab4*, a well characterized nuclear gene encoding a chloroplast protein synthesized in the cytoplasm¹⁷ (Fig. 3).

The evolutionary relationship between the *Arabidopsis* nuclear *tufA* and known chloroplast *tufA* genes was investigated by phylogenetic analysis using amino-acid sequences of EF-Tu and EF-1 α , the eukaryotic and archaeobacterial homologue of EF-Tu. In the resulting tree (Fig. 4) the *Arabidopsis* EF-Tu is found nested within a clade of chloroplast-encoded EF-Tus. This group is in turn the sister group to a clade containing the EF-Tu of the cyanobacterium *Anacystis*, which is consistent with the proposed origin of chloroplasts from endosymbiotic cyanobacteria. Thus the *Arabidopsis* nuclear *tufA* seems to be derived from a green algal chloroplast gene.

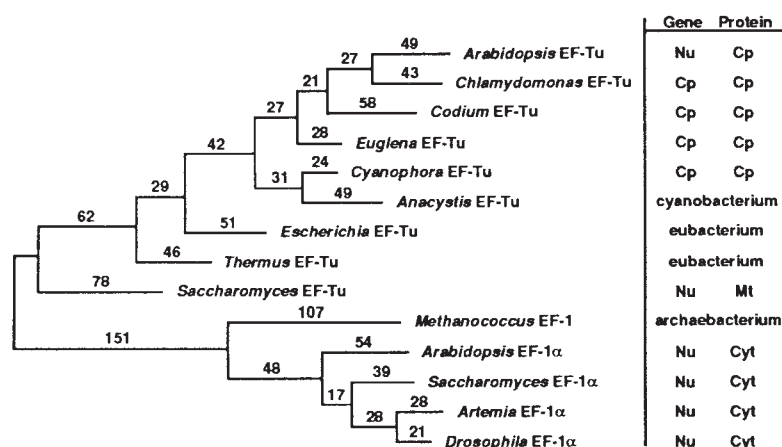
The *Chlamydomonas* and *Arabidopsis tufAs* seem to be the sole functional *tufA* in their respective organisms. Each is the only detectable cellular copy of the gene (Fig. 1; ref. 18) and is actively transcribed (Fig. 3; ref. 19). The simplest explanation for the subcellular distribution of the gene is that *tufA* was transferred from the chloroplast to the nucleus. This conclusion is also further supported by phylogenetic inference (Fig. 4).

Alternatively, it could be argued that ancestral *tufA* was present in the nucleus and then transferred to the chloroplast, but this would necessitate at least four independent nuclear-to-chloroplast gene transfers. Each of these transfers would also entail the precise removal of the transit peptide sequence, because the predicted amino termini of all eubacterial and chloroplast-encoded EF-Tus align precisely. The origination of the *Arabidopsis* nuclear *tufA* gene as a result of substitution of a pre-existing nuclear *tufA*-like gene of non-chloroplast origin, such as one encoding a cytoplasmic EF-1 α or mitochondrial EF-Tu, is ruled out by overall sequence comparison and by the gene phylogeny (note the disparate positions of the *Arabidopsis* EF-1 α and EF-Tu proteins in Fig. 4).

We have found a chloroplast *tufA* in representative ulvophyceae, chlorophyceae and charophyceae green algae (Fig. 2; S.L.B., J. R. Manhart, M. Kuhse and J.D.P., unpublished results), and its absence from the chloroplast chromosome of a liverwort has been established by complete sequencing of that genome¹³. The generally accepted phylogeny of green plants places the Charophyceae as the algal lineage giving rise to land plants, and liverworts as the earliest branch within land plants²⁰. We therefore propose that *tufA* was ancestrally present in the green algal chloroplast, retained in each of the main lineages after their separation, and then transferred to the nucleus within the charophyceae lineage before the emergence of land plants. This places the *tufA* transfer at 450–500 million years ago²¹.

The two other cases that are indicative of modern evolutionary gene transfer are, in fact, both ambiguous. The evolutionary history of *rbcS*, which is nuclear-encoded in land plants and green algae but chloroplast-encoded in all other algae²², is confused by uncertainty regarding common or separate endosymbiotic ancestry for the chloroplasts of the different algal groups. The *atp9* gene is encoded in the mitochondrion in yeast but by the nucleus in *Neurospora*, with *Neurospora* also carrying an apparently silent *atp9* gene in its mitochondrion²³. However, sequence similarity between the two *Neurospora* genes is low, indicating that these are probably not orthologous, and the low similarity between *Neurospora* genes and the yeast *atp9*, combined with the small size of the gene, all make it difficult to trace its evolutionary history²³ (S.L.B., unpublished data). Further differences in gene content have also been noted among the three completely sequenced chloroplast genomes^{10,13}, and among mitochondrial genomes of the principal groups of

FIG. 4 The *Arabidopsis* nuclear *tufA* encodes a chloroplast-type EF-Tu. The tree shown is the single shortest tree found by cladistic analysis of fourteen DNA-derived EF-Tu and EF-1 α amino acid sequences and has a total length of 1,158 steps and a consistency index of 0.80 excluding autapomorphies. Rooting is at the midpoint, and horizontal branches are drawn proportional to their lengths, which are indicated numerically above the branches. The table on the right shows the coding compartment (Gene) and cellular site of activity (Protein) for each protein included in the tree. Eukaryotic cellular compartments are indicated as chloroplastic (cp), mitochondrial (mt), nuclear (nc) or cytoplasmic (cyt), whereas non-eukaryotic proteins are identified as such. Sources of EF-Tu/EF-1 α sequences in addition to the two reported in this paper: *Anacystis nidulans*¹⁵, *Arabidopsis thaliana* nuclear-cytoplasmic³⁰, *Artemia salina*³¹, *Codium fragile* (M. Kuhsel, unpublished results), *Cyanophora paradoxa* (M. Kraus & W. Loeffelhardt, unpublished results), *Drosophila melanogaster*³², *E. coli*¹⁶, *Euglena gracilis*¹², *Methanococcus vanellii*³³, *Saccharomyces cerevisiae* mitochondrion³⁴, *Saccharomyces cerevisiae* nuclear-cytoplasmic³⁵, and *Thermus thermophilus*³⁶. METHODS. Deduced amino-acid sequences were aligned³⁷ with the Multalign program of the Molecular Biological Information Resource package EuGene Release 3.2 (Lark Sequencing Technologies Ltd, Houston, Texas) on a SUN computer running Unix 4.0.3. Default penalties were used for gaps, with minor adjustments made by eye. Cladistic analysis was performed using



the branch and bound option of PAUP version 2.4.2 (D. Swofford, Illinois Natural History Survey, Champaign, Illinois) on the SUN computer. Amino acids were scored for all informative sites as equally weighted, unordered, multistate characters. Gaps were scored as missing data and were not used as characters in the analysis.

eukaryotes⁴. Further study will determine which of these coding differences involve gene transfer, gene substitution, or gene loss.

The movement of functional genes must require the fortuitous combination of physical relocation of a complete gene, insertion into a favourable nuclear locus, and acquisition of expression signals and an in-frame transit sequence, all within a relatively short time to prevent mutational loss of function. This could explain why functional transfer is so rare compared with the physical transfer of non-functional organelle sequences to the nucleus^{7,8}, and would also predict that physical transfer of a gene will not occur simultaneously with the loss of its organellar copy. Characterization of other physical and functional gene transfers may elucidate the mechanisms involved in intracellular DNA movement and in the acquisition of nuclear-expression and organelle-targeting signals. □

35. Nagata, S. *et al.* *EMBO J.* **3**, 1825–1830 (1984).

36. Seidler, L., Peter, M., Meissner, F. & Sprinzl, M. *Nucleic Acids Res.* **15**, 9263–9277 (1987).

37. Feng, D.-F. & Doolittle, R. F. *J. molec. Evol.* **25**, 351–360 (1987).

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Peptide mimetics of an actin-binding site on myosin span two functional domains on actin

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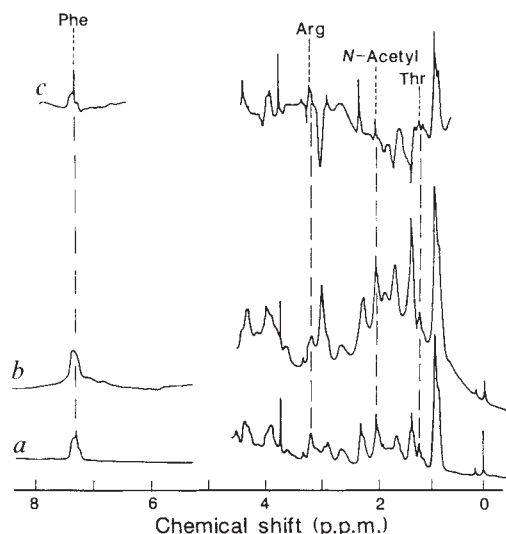
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THE sites on the myosin heavy chain that interact with actin and are responsible for force generation are ill-defined: crosslinking^{1,2} and experiments with isolated domains of the myosin head^{3,4} implicate regions in both the 50K and 20K (molecular weights in thousands) domains of the myosin head (subfragment 1, S1) in this process. We have synthesized peptides from the sequence around the fast-reacting SH₁ thiol residue in the 20K domain of S1 in order to delineate precisely an actin-binding site. We used a combination of ¹H-NMR and enzyme inhibition assay and also assessed the effects of peptides on skinned rabbit psoas muscle fibres to show that the region of amino acids 690–725 contains an actin-binding site. Peptides from this region bind to actin, act as mixed inhibitors of the actin-stimulated S1 Mg²⁺-ATPase, and influence the contractile force developed in skinned fibres, whereas peptides flanking this sequence are without effect in our test

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- Gray, M. W. *Trends Genet.* **5**, 294–299 (1989).
- Shih, M.-C., Lazar, G. & Goodman, H. M. *Cell* **47**, 73–80 (1986).
- Palmer, J. D. A. *Rev. Genet.* **19**, 325–354 (1985).
- Attardi, G. & Schatz, G. A. *Rev. Cell Biol.* **4**, 289–333 (1988).
- Tingey, S. V., Tsai, F.-Y., Edwards, J. W., Walker, E. L. & Coruzzi, G. M. *J. biol. Chem.* **263**, 9651–9657 (1988).
- Surguchov, A. P. *Trends biochem. Sci.* **12**, 335–339 (1987).
- Timmis, J. N. & Scott, N. S. *Nature* **305**, 65–67 (1983).
- Gellissen, G. & Michaelis, G. *Ann. N.Y. Acad. Sci.* **503**, 391–401 (1988).
- Nagley, P. & Devenish, R. J. *Trends biochem. Sci.* **14**, 31–35 (1989).
- Sugiura, M. A. *Rev. Cell Biol.* **5**, 51–70 (1989).
- Watson, J. C. & Surzycki, S. J. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2264–2267 (1982).
- Montandon, P.-E. & Stutz, E. *Nucleic Acids Res.* **11**, 5877–5891 (1983).
- Ohyama, K. *et al.* *Nature* **322**, 572–574 (1986).
- Keegstra, K. & Olsen, L. J. A. *Rev. Plant Physiol. Plant molec. Biol.* **40**, 471–501 (1989).
- Meng, B. Y., Shinozaki, K. & Sugiura, M. *Molec. gen. Genet.* **216**, 25–30 (1989).
- Yokota, T., Sugisaki, H., Takamami, M. & Kaziyo, Y. *Gene* **12**, 25–31 (1980).
- Tobin, E. M. & Silverthorne, J. A. *Rev. Plant Physiol.* **36**, 569–593 (1985).
- Watson, J. C. & Surzycki, S. J. *Curr. Genet.* **7**, 201–210 (1983).
- Silk, G. W. & Wu, M. *Curr. Genet.* **14**, 119–126 (1988).
- Bremer, K., Humphries, C. J., Mishler, B. D. & Churchill, S. P. *Taxon* **36**, 339–349 (1987).
- Stewart, W. N. *Paleobotany and the Evolution of Plants* (Cambridge University Press, 1984).
- Douglas, S. E. & Durnford, D. G. *Plant molec. Biol.* **13**, 13–20 (1989).
- van den Boogaart, P., Samallo, J. & Agsteribbe, E. *Nature* **298**, 187–189 (1982).
- Watson, J. C. & Thompson, W. F. *Meth. Enzym.* **118**, 57–75 (1986).
- Palmer, J. D. *Meth. Enzym.* **118**, 167–186 (1986).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
- Henikoff, S. *Gene* **28**, 351–359 (1984).
- Lizardi, P. M. *Meth. Enzym.* **96**, 24–38 (1983).
- Jost, J.-P. & Pehling, G. *Eur. J. Biochem.* **66**, 339–346 (1976).
- Axelos, M. *et al.* *Molec. gen. Genet.* **219**, 106–112 (1989).
- Lenstra, J. A., Van Vliet, A., Arnberg, A. C., Van Hemert, F. J. & Molter, W. *Eur. J. Biochem.* **155**, 475–483 (1986).
- Walldorf, U., Hovemann, B. & Bautz, E. K. F. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5795–5799 (1985).
- Lechner, K. & Bock, A. *Molec. gen. Genet.* **208**, 523–528 (1987).
- Nagata, S., Tsunetsugu-Yokota, Y., Naito, A. & Kaziyo, Y. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6192–6196 (1983).

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systems. Remarkably, peptides from the N-terminal half of this segment 690–725 increase force development in skinned fibres at submaximal activating concentrations of Ca^{2+} , that is, they behave as calcium-sensitizers; C-terminal peptides, however, inhibit force development without effecting sensitivity to calcium. These different responses indicate that this region is probably binding at two functionally distinct sites on actin.

Our ideas focused on the region around the SH_1 thiol when it was observed that a nitroxyl-spin label attached to this group perturbed ¹H-NMR resonances specifically arising from the N-terminal region of actin (Fig. 1), indicating that the two loci must be close (≤ 1.5 nm) in the actin–SI complex. To define the amino acids involved at this interface, we synthesized peptides spanning this region on SI (Fig. 2). Although we did not expect these peptides to assume their SI structure in solution, we anticipated that they contained sufficient information to form the structure in the presence of a suitable template, namely actin. We found that peptides containing all or a portion of the residues from 690–725 act as mixed inhibitors⁵ of the actin–SI Mg^{2+} -ATPase activity, with inhibition constant (K_i) values ranging from 5 μM (for Y630, a long peptide that spans most of this region) to 340 μM (for Y668, a 10-residue peptide containing a portion of the N-terminal end of this region). Figure 3a shows a typical kinetic plot used to derive K_i , and in Fig. 3b values of K_i (as $\log 1/K_i$) are plotted against the sequence. Note that peptides from outside the region 690–725 are not inhibitory and act as controls. Direct binding assays in which the peptides are co-sedimented with F-actin confirm that 1 mol peptide is bound per mol of actin and that values of the dissociation constant (K_d) agree closely with those for K_i .

We also tested these peptides for their ability to influence the calcium activation of the regulated actin complex (actin +

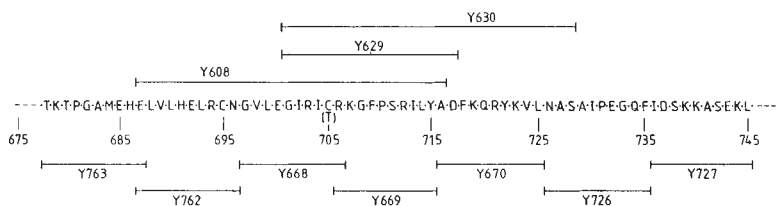
FIG. 1 The binding of SI(A2) spin-labelled at Cys 705 to the N-terminal CNBr-fragment of actin (residues 1–44). a, ¹H-NMR spectrum of actin peptide (residues 1–44), 150 μM; b, ¹H-NMR spectrum of actin 1–44 (150 μM) and spin-labelled SI(A2) (63 μM); c, Difference spectrum, a – b, showing residues perturbed by the spin label. Addition of dithionite (1 mM) to b reduces the spin label, so removing its paramagnetic properties, and diminishes the resulting difference spectrum (data not shown). This indicates the proximity to the N-terminal peptide of actin of the SH_1 thiol on SI. We used F-actin in a similar experiment and obtained identical results.

METHODS. SI was prepared from rabbit skeletal myosin by chymotryptic digestion and the light chain isoenzymes (SI(A1) and SI(A2)) separated by ion-exchange chromatography on SP-Tris Acryl (LKB). The fast-reacting SH_1 thiol group (Cys 705) was covalently reacted with 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl¹². The N-terminal CNBr fragment from actin (residues 1–44) was provided by A. J. G. Moir (University of Sheffield). It was freeze-dried once from ²H₂O and dissolved in 10 mM phosphate buffer, pH 7.1 (uncorrected), containing 0.02% NaNO_3 and 0.1 mM sodium 3-trimethylsilylpropionate in ²H₂O. Spin-labelled SI(A2) was prepared for spectroscopy as before¹² and dialysed against several changes of the ²H₂O-phosphate buffer. ¹H-NMR experiments were performed on a 300 MHz Bruker spectrometer operated in the Fourier-transform mode. Typical spectral accumulation times were 20–30 min and measurements were made at 297 K. Spectral perturbations resulting from complex formation were monitored by difference spectroscopy (as in c), and by the application of two-pulse ($180-\tau-90-\tau$) spin-echo methods (data not shown).

tropomyosin + troponin) stimulation of SI Mg^{2+} -ATPase activity. In Fig. 3c, which shows the effect of a selection of peptides on the (normalized) pCa/ATPase relationship, two effects are apparent. Peptides from the N-terminal end of this region (containing residues ~690–715), such as Y668 and Y669, shift the pCa-activity relation significantly to the left and therefore act as calcium-sensitizers, whereas peptides from the C-terminal end (~716–728), for example Y670, have very little, if any, calcium-sensitizing activity. It should be noted that Y670 binds to actin seven times more strongly than Y668 (Fig. 3b, showing that the leftward shift in the pCa curve is not a function of tightness of binding. In all, the data in Fig. 3 illustrate the specificity of actin binding of the region around 690–725 of the myosin heavy chain and indicate that at least a portion of this segment could be involved in the Ca^{2+} -regulation process.

We next tested the influence of our peptides on force generation in rabbit psoas fibres chemically skinned with 1% Triton X-100. Peptides (final concentrations, 20–100 μM) were added to a bath containing the fibres, which were then activated with Ca^{2+} . Peptides Y608, Y629, Y668, Y669 and Y762 all increase the isometric tension elicited at ~2 μM Ca^{2+} in a dose-dependent manner and cause a left-handed shift in the calcium-force relationship (Fig. 4). Their effects are reversed on washing out the peptide (Fig. 4a). Note that these peptides all contain only the N-terminal half of the sequence and the data agree with those in Fig. 3c. On the other hand, peptide Y670 from the C-terminal end is weakly inhibitory (Fig. 4c), and peptide Y630, which contains most of this region, strongly inhibits force development and its effects were also partially reversible (Fig. 4a, b). This suggests that inhibition by peptides from the C-terminal end might dominate the properties of the whole region, possibly by competing with SI for (one of) the principal sites

FIG. 2 Peptides synthesized from the SH_1/SH_2 region of the 20K domain of rabbit fast skeletal myosin. The sequence of the region 675–745 of the 20K domain of myosin is shown encompassing the fast-reacting thiol groups, SH_1 and SH_2 (numbered 705 and 695, respectively). The peptides, of lengths indicated by the bars, were synthesized by the conventional solid phase route by Alta Bioscience, University of Birmingham. The 600 series of peptides were purified by a combination of gel filtration and ion exchange chromatography on SP-TrisAcryl. The 700 series were purified by HPLC on semi-preparative Vydac C-18 columns with 300 Å pore size (Technicol, Stockport, UK). The purity of all peptides was checked by analytical HPLC and by amino-acid analysis. The concentration of peptide solutions was also determined by amino-acid analysis. In peptide Y608, Cys



705 was replaced by Thr (as is found in the *Dictyostelium discoideum* myosin sequence) to prevent excessive oxidation and to facilitate purification.

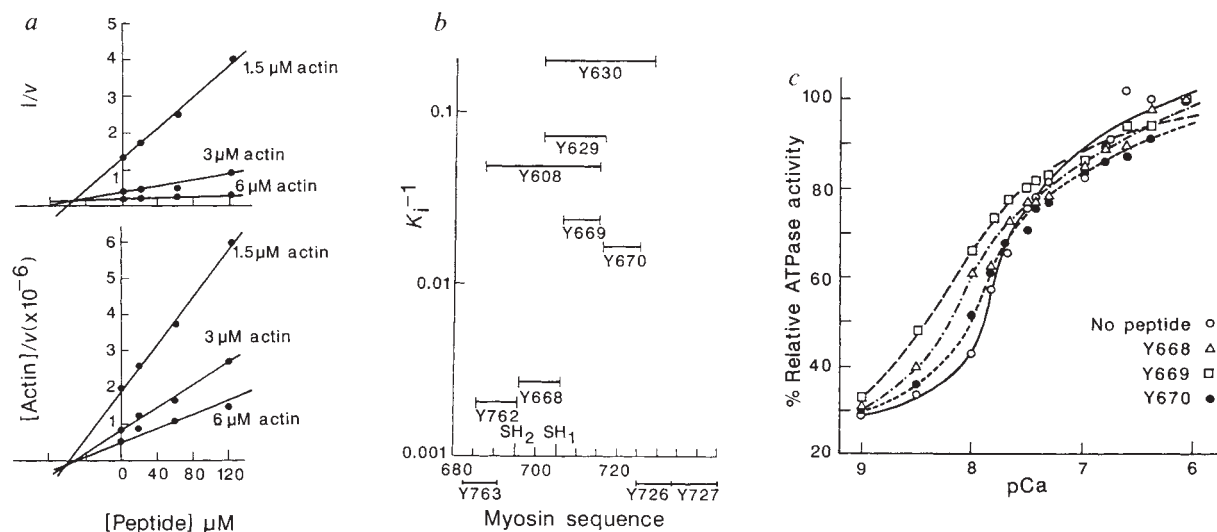


FIG. 3 Effects of the peptides on actin-SI Mg^{2+} -ATPase activity. Steady-state ATPase activity was monitored by inorganic phosphate liberated¹³ as a function of time in a total assay volume of 0.25 ml at 25 °C. Assays were in 25 mM triethanolamine-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 5 mM ATP with 0.6 μM SI. *a*, Determination of K_i from plots of $1/\text{initial rate}$ against inhibitor (peptide) concentration¹⁴, and of K_i' from plots of $actin/\text{initial velocity}$ against inhibitor (peptide) concentration¹⁵ for peptide Y670. In all cases where the peptides are inhibitory, the point of intersection is just above the axis in the upper plot and just below it in the second, indicating mixed inhibition⁵. Derived K_i values were always about equal to K_i' values. *b*, K_i values derived from *a* are plotted as $\log 1/K_i$ against the myosin sequence. K_i values varied from 5 μM (for Y630) to 340 μM (for Y668).

Bars below the horizontal axis indicate peptides having no effect on actin-SI Mg^{2+} -ATPase activity. *c*, The relative ATPase activity of the regulated actin-SI Mg^{2+} -ATPase is plotted against $-\log [Ca^{2+}]$. The tropomyosin-troponin mixture was prepared¹⁶, mixed with actin (3 μM actin, 0.86 μM tropomyosin-troponin per assay) and assayed in the presence of 60 μM peptide. Buffered Ca^{2+} ion concentration was adjusted by addition of calcium EGTA buffer (3 mM) and was determined from the ratio of EGTA to calcium-chelated EGTA as described in the legend to Fig. 4. ATPase values for all curves were normalized to 100% at pCa 6.1. Data are the average of 4 or 5 determinations on at least two different protein preparations. The scatter around each point was always less than $\pm 5\%$ of the average. For the sake of clarity, error bars are not included.

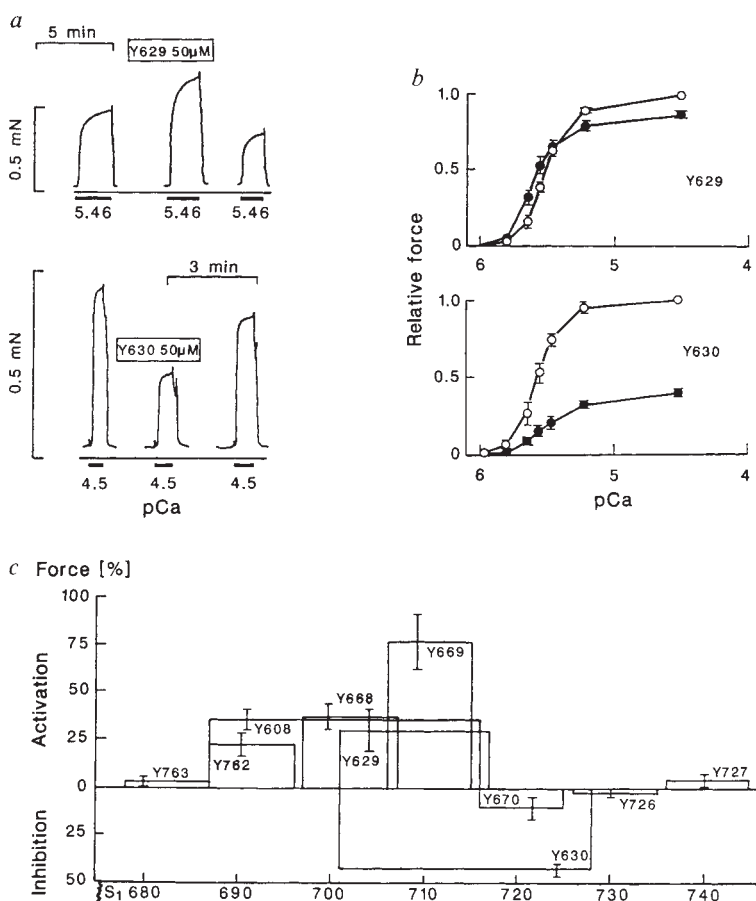


FIG. 4 Effect of peptides on tension responses of skinned fibres. *a*, Upper: activating effect of peptide Y629 (50 μM) on force development of skinned fibres submaximally activated at pCa 5.5. Lower: Inhibitory effect of peptide Y630 (50 μM) on force development of skinned fibres (maximally) activated at pCa 4.5. *b*, pCa -force relationship of skinned psoas fibres before (open circles) and after addition (filled circles) of 50 μM peptide. Values are the averages $\pm$ s.e. of 6 and 5 experiments. Note the increase in calcium sensitivity (pCa_{50} values) after addition of peptide Y629 and the slight but significant inhibition at maximal calcium activation. The pCa -activity relationship is shifted towards higher Ca concentrations in skinned fibres compared with the regulated actin-SI Mg^{2+} -ATPase. This is due to the different conditions of lower pH, higher ionic strength, and the presence of P_i ^{17,18}. *c*, Activity profile of synthetic peptides corresponding to peptide fragments from the 20K domain of myosin SI (residue numbers on abscissa). Activation or inhibition of force development of skinned fibres is indicated as per cent of control contraction before addition of the peptide elicited at pCa = 5.6 (average $\pm$ s.e. of 5-6 experiments). Note that a biologically active peptide region is flanked by an inactive region.

METHODS. After mounting the preparations with a fast-setting glue on an isometric force transducer (AME 801, SensoNor, Horten, Norway) and a glass rod attached to a micromanipulator, fibres were relaxed by immersion into a solution containing (in mM): imidazole, 30; ATP, 10; creatine phosphate, 10; $NaNO_3$, 5; calcium EGTA, 5; $MgCl_2$, 12.5; creatine kinase 380 U ml^{-1} ; dithiothreitol, 5; P_i , 5. Ionic strength was adjusted to 80 mM with KCl. Contraction was induced by immersion into an analogous solution in which EGTA was replaced by a calcium EGTA buffer. The buffered calcium ion concentration was determined from the ratio of EGTA to calcium EGTA, essentially according to ref. 20, but using an apparent dissociation constant of 1.6 μM for the calcium EGTA buffer at pH 6.7 and 20 °C (compare ref. 21).

on actin responsible for the energy transduction process. In Fig. 4c, we illustrate these effects by plotting force increase or inhibition by these peptides (at 50 μM) as a percentage of control force at 2.5 μM Ca^{2+} in the absence of peptide. In this way, an activity profile is obtained over the stretch of SI peptide comprising residues 680–745 (shown on the abscissa), which also includes the inactive flanking regions.

It should be emphasized that at maximally activating concentrations of Ca^{2+} , all the peptides inhibit force development to some degree (see Fig. 4b for example); this is to be expected should they be interfering with the actomyosin interaction, as we have noted in the actin–SI Mg^{2+} -ATPase solution assays (Fig. 3a, b). In skinned fibres, however, peptides from the N-terminal region (Y608, Y629, Y668, Y669 and Y762) are only slightly inhibitory ($\sim 15\%$; Fig. 4b, upper panel), but this effect is accompanied by an increase in force development at submaximal activating concentrations of calcium which is significant compared with controls ($32 \pm 10\%$ in the case of peptide Y629 for example; Fig. 4). It is tempting to explain the enhancement of force at submaximal calcium concentrations in terms of a reduction of the apparent crossbridge-detachment rate constant (compare ref. 6). If this were the case, however, the force developed at maximal calcium concentration would have to increase rather than decrease. A dual effect of the peptides would be more feasible if they were competing with both troponin-I and myosin for their respective binding sites on actin.

We confirmed that the synthetic peptides affect both ATPase and contractile activity by their direct interaction with actin, using ^1H -NMR to monitor binding to actin. These experiments show that the peptides with biological activity bind to actin, whereas those from the flanking sequences do not. They also indicate that only selected amino-acid side chains or groups of amino acids along this sequence are perturbed by actin-binding. This will be commented on more fully elsewhere in the light of NMR data on the interaction of actin and troponin-I (ref. 7). Morita and colleagues⁸ also reported, while these experiments were in progress, that a small peptide from this region (residues 702–708) can interfere with SI binding to actin. We cannot be sure that this is the only site on the 20K domain that is involved in actin binding, and indeed crosslinking studies may indicate otherwise⁹.

Our results, however, indicate that the region 690–725 on the myosin head is likely to span two functionally distinct (and possibly spatially distinct) sites on actin in the actin–myosin complex. The crossbridges attaching to actin are known to increase the calcium sensitivity of the contractile system¹⁰. It is therefore plausible that the calcium-sensitizing effects of the N-terminal region could result from this segment weakening the actin–troponin-I interaction, either by direct competition with troponin-I for the same site on actin (both troponin-I (ref. 7) and SI (refs 1, 2) bind to the N-terminal region of actin) or as a result of some allosterically transmitted event. In either case, the peptides would mimic the ‘potentiating effect’ of SI on actin¹¹ and switch on the thin filament. The C-terminal half, whose inhibitory effects on force overshadow these calcium-sensitizing properties, should then bind at another site on actin, giving rise to the idea of a multi-site docking of the interacting partners in the force-generating cycle, an idea implicit in many of the crosslinking studies^{1,2,9}. This could arise from the blocking of the actin–SI interaction directly, or from switching off the thin filament by some other means. We are using these peptides as specific inhibitors of interacting actin–myosin sites to investigate the kinetic stages of the contractile event and to relate these more precisely with molecular events. □

4. Mulrad, A., Kasprzak, A. A., Ue, K., Atjai, K. & Burghart, T. P. *Biochim. biophys. Acta* **869**, 128–140 (1986).
5. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics* (Butterworths, London, 1979).
6. Brenner, B. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3265–3269 (1988).
7. Levine, B. A., Moir, A. J. G. & Perry, S. V. *Eur. J. Biochem.* **172**, 389–397 (1988).
8. Suzuki, R., Nishi, N., Tokura, S. & Morita, F. *J. Biol. Chem.* **262**, 11410–11412 (1987).
9. Sutoh, K. *Biochemistry* **21**, 4860–4804 (1982).
10. Guth, K. & Potter, J. D. *J. Biol. Chem.* **263**, 13627–13635 (1987).
11. Weber, A. & Murray, J. M. *Physiol. Rev.* **53**, 612–673 (1973).
12. Trayer, I. P., Trayer, H. R. & Levine, B. A. *Eur. J. Biochem.* **164**, 259–266 (1987).
13. Fiske, C. H. & Subbarow, Y. *J. Biol. Chem.* **66**, 375–380 (1925).
14. Dixon, M. *Biochem. J.* **55**, 161–170 (1953).
15. Cornish-Bowden, A. *Biochem. J.* **137**, 143–144 (1974).
16. Eisenberg, E. & Kielly, W. W. *J. Biol. Chem.* **249**, 4742–4748 (1974).
17. Blanchard, E. M., Bo-Sheng, P. & Solaro, R. J. *J. Biol. Chem.* **259**, 3181–3186 (1984).
18. Brandt, P. W., Cox, R. N., Kawai, M. & Robinson, T. *J. gen. Physiol.* **79**, 997–1016 (1982).
19. Kawai, M. & Schulman, M. I. *J. Muscle Res. Cell Mot.* **6**, 313–332 (1985).
20. Fabiato, A. & Fabiato, F. *J. Physiol., Paris* **75**, 463–505 (1979).
21. Blinks, J. R., Weir, W. G., Hess, P. & Prendergast, F. G. *Prog. biophys. molec. Biol.* **40**, 1–114 (1982).

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Relative helix-forming tendencies of nonpolar amino acids

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AN important issue in understanding the relationship between protein sequence and structure is the degree to which different amino acids favour the formation of particular types of secondary structure. Estimates of the ‘helix-forming tendency’ of amino acids have been made based on ‘host–guest’ experiments, in which copolymers are made of the amino acid of interest (the ‘guest’) and a host residue (typically hydroxypropyl- or hydroxybutyl-L-glutamine)¹. Recently, however, short alanine-based peptides were found to form stable monomeric helices in water², contrary to the result predicted from host–guest experiments². We have now measured the helix-forming tendency of five different nonpolar amino acids (Ala, Ile, Leu, Phe, Val) by substituting each in turn for alanine in a 17-residue alanine-based peptide and determining the extent of α -helix formation. Our results differ from those of host–guest experiments both in the degree of variation in helix-forming tendency of different amino acids, and in the rank order of the helix-forming tendency. We conclude that the helix-forming tendency of a particular amino acid depends on the sequence context in which it occurs; and the restriction of side-chain rotamer conformations is important in determining the helix-forming tendency.

We measured the rank order of helix-forming tendency of five nonpolar amino acids by substitution experiments using a 17-residue alanine-based peptide as a reference. Our three underlying objectives were: (1) to determine whether other nonpolar amino acids share with Ala the ability to form an α -helix in short peptides; (2) to determine whether the results of amino-acid substitution experiments are consistent with predictions from host–guest results; and (3) to find the factors that determine the helix-forming tendency of a nonpolar amino acid. We have deferred the study of other amino acids, because polar amino acids can disrupt the helix by hydrogen-bonding to main-chain NH or CO groups, and charged amino acids have additional complications imposed by electrostatic interactions.

The reference peptide that we used for the substitution experiments (Table 1) contained four Lys⁺ residues inserted for peptide solubility in water, and one Tyr residue (N-terminal) to allow us to determine peptide concentration accurately by

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1. Morinet, D., Bertrand, R., Pantel, P., Audemard, E. & Kassab, R. *Biochemistry* **20**, 2110–2120 (1981).

2. Sutoh, K. *Biochemistry* **22**, 1579–1585 (1983).

3. Chaussepied, P. et al. *Biochemistry* **25**, 4540–4547 (1986).

TABLE 1 Peptide sequences and helix-forming properties

Peptide	Sequence* $\parallel$	$-[\theta]_{222}^{\dagger}$	Residue	% Helix $\ddagger$	$s^{\S}$	$P\alpha^{\parallel}$
3Ala	CH ₃ CO-YKAAAKAKAAAKAAAK-NH ₂	25,800	Ala	78	1.08	1.60
3Leu	CH ₃ CO-YKAALAKAALAKAALAK-NH ₂	26,300	Leu	80	1.10	1.50
3Phe	CH ₃ CO-YKAAFAKAAFAKAAFAK-NH ₂	7,600	Ile	41	1.25	1.31
3Ile	CH ₃ CO-YKAAIAKAAIAKAAIAK-NH ₂	13,400	Phe	23	1.06	1.45
1Val	CH ₃ CO-YKAAAKAAVAKAAAK-NH ₂	16,200	Val	17	0.88	1.09
2Val	CH ₃ CO-YKAAVAKAAVAKAAAK-NH ₂	11,200				
3Val	CH ₃ CO-YKAAVAKAAVAKAAVAK-NH ₂	5,600				

* The α -NH₂ is blocked by an acetyl group, and the α -COOH is blocked by an amide group.

$\dagger$ Mean residue ellipticity at 222 nm. Conditions: 0 °C, pH 7.0, 1.0 M NaCl, 1 mM each sodium citrate, sodium phosphate and sodium borate. Units: degrees cm² dmol⁻¹.

$\ddagger$ The % helix is taken to be proportional to $-[\theta]_{222}$. The values for 100 and 0% helix are 33,000 and 0 degrees cm² dmol⁻¹ (see text). Values for peptides with three residue substitutions (except for Ala) are shown.

$\S$ Host-guest values for s (taken from refs cited in ref. 5) at 0 °C, based on experiments with random sequence copolymers in which the host residue is hydroxypropyl-L-glutamine (HPLG) or hydroxybutyl-L-glutamine; s is the helix propagation parameter in the helix-flexible chain transition theory.

$\parallel$ $P\alpha$ is the helical preference⁶ given by the frequency of an amino acid in α -helices (the three residues at each end are excluded), relative to its frequency in the entire protein.

$\parallel$ Peptide synthesis: Peptides were synthesized by solid-phase peptide synthesis on an automated Milligen 9050 synthesizer, using standard 9-fluorenylmethoxycarbonyl methodology¹³. The N terminus was acetylated with acetic anhydride and the peptides were cleaved from the benzhydrylamine resin by incubation with 95:5 trifluoroacetic acid:phenol mixture for at least 2 h yielding an amide at the C terminus. Peptide Purification: Peptides were purified by gel filtration and reverse phase chromatography as previously described². Peptide identity was verified by mass spectrometry.

measuring tyrosine absorbance². We substituted the chosen non-polar amino acid for Ala at one to three positions (Table 1) spaced at least five residues apart to minimize intrahelical interactions between the substituted nonpolar residues. The 17-residue peptides were in the same size range as those of helical segments found in globular proteins, and the results show how differences in helix-forming tendency of an amino acid can affect the stability of a short helix.

Circular dichroism (CD) spectra of the peptides measured at 0 °C in 0.1 M KF, pH 7, are shown in Fig. 1. With the exception of the peptide in which three Ala residues had been substituted by Phe (peptide 3Phe), all the peptides have CD spectra expected for mixtures of α -helix and random chain, with a first minimum at 222 nm, a second minimum between 197 and 207 nm, and a

maximum at about 190 nm. The helix content (% helix) is taken as directly proportional to the mean residue ellipticity at 222 nm, $-[\theta]_{222}$. The existence of an isodichroic point (203 nm) is consistent with the presence of just two conformations for each residue, helix and random chain. The spectrum of 3Phe is anomalous; it does not contain the isodichroic point shown by the other peptides, and its value for % helix derived from $-[\theta]_{222}$ is tentative. We determined the value of $-[\theta]_{222}$ corresponding to 100% helix from the maximum value given by trifluoroethanol (TFE) titration⁴ at 0 °C (data not shown). The same maximum value, within the limits of experimental error, was found for each peptide: $33,000 \pm 1,000$ degrees cm² dmol⁻¹.

The extent of helix formation in similar alanine-based peptides is independent of peptide concentration throughout the helix/random chain transition². Figure 2 shows that the reference peptide, 3Ala, is within the limits of experimental error, monomeric in the concentration range tested (up to 6 mg ml⁻¹), as judged by measurements of its apparent relative molecular mass (M_r) in sedimentation equilibrium experiments, using both Rayleigh interference and light absorption optical systems.

Table 1 shows the helix content at 0 °C, pH 7, for all the peptides studied. The peptide 3Leu forms a stable α -helix, comparable to 3Ala. Only leucine shares the ability of alanine to support α -helix formation in this short-peptide system. Ile, Val and Phe are helix destabilizing relative to Ala. Results for the series of peptides 1Val, 2Val and 3Val indicate that peptides with ≥ 4 Ala $\rightarrow$ Val substitutions would abolish measurable helix formation.

Table 1 shows that the rank order of helix-forming ability is clearly different from that predicted by host-guest values of s (ref. 5), the helix propagation parameter. The host-guest values of s for Ala, Leu and Phe are the same, within the limits of experimental error, and s for Ile is higher than it is for Ala (Table 1), whereas in our alanine-based peptide system both Phe and Ile are definitely helix-destabilizing compared with Ala and Leu. To compare the changes in helicity with predictions based on host-guest data, we used an approximate two-state model (see legend to Fig. 3), which gives an upper limit to the change expected in a substitution experiment. The changes that we observed for Ile, Val and Phe were large compared with this predicted upper limit. Consequently, the differences in helix-forming tendency that we measured are much larger than those predicted from host-guest values of s . Whether the differences in helix-forming tendency are large (the results presented here) or small (host-guest results) is important in determining whether local sequence effects or tertiary interactions are dominant in determining α -helix locations in proteins. Our results indicate that the helix-forming tendencies of the amino acids contribute

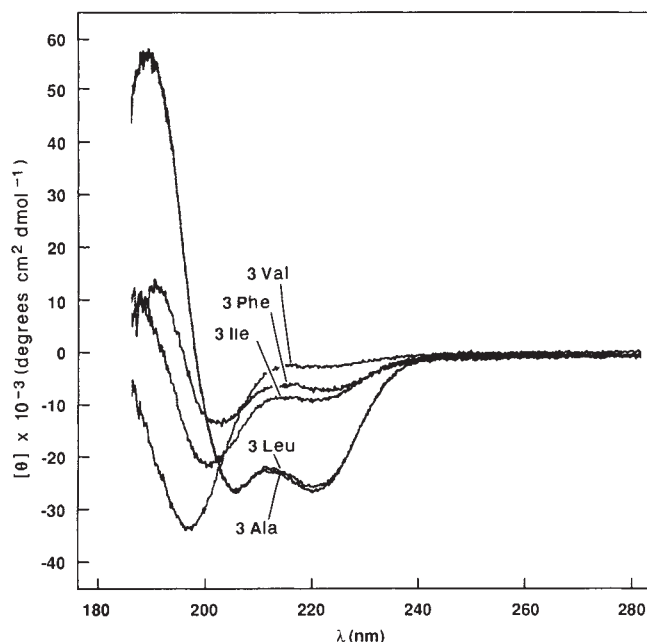


FIG. 1 CD spectra of the peptides taken at 0 °C, 0.1 M KF, 1 mM KPO₄, pH 7.0. The mean residue ellipticity (degrees cm² dmol⁻¹) is plotted. In order of decreasing values of $-[\theta]_{222}$, the spectra are for peptides 3Leu, 3Ala, 3Ile, 3Phe and 3Val (see Table 1).

METHODS. CD spectra were taken on an Aviv 60DS spectro-polarimeter. Samples were prepared as described². The spectra shown above were taken at a peptide concentration of 50 μ M in a 1-mm path length cuvette. Data were taken with a 0.2-nm step size, 1-s average time, and the results averaged over four scans. Identical spectra were obtained in a 1-cm path length cuvette with a 15 μ M peptide concentration.

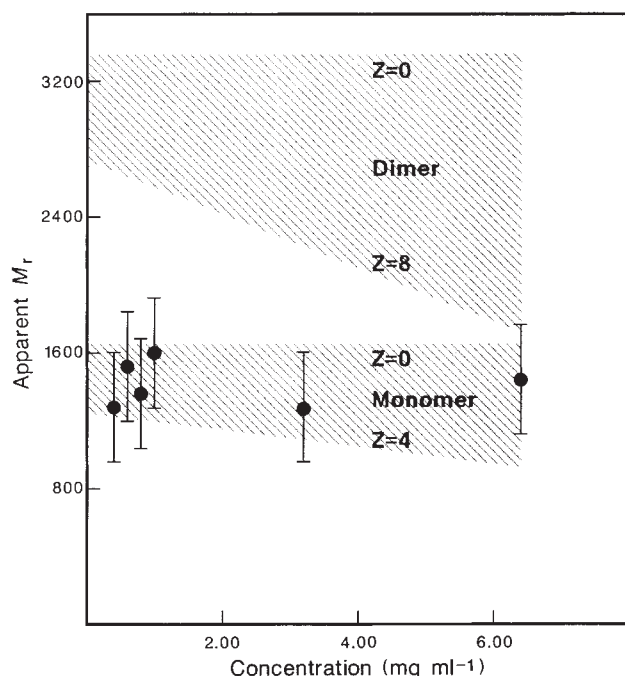


FIG. 2 Concentration dependence of the apparent M_r of peptide 3Ala (Table 1), measured by sedimentation equilibrium¹⁴ using both Rayleigh interference and absorbance (275 nm) optical systems, at 20.0 °C, in 0.1 M NaCl, and 1 mM sodium citrate, phosphate and borate, pH 7.0. The partial specific volume was calculated¹⁵ from peptide composition to be 0.76 ml g⁻¹; the measured buffer density was 1.0021 g ml⁻¹, and NONLIN¹⁶ was used to fit the sedimentation results. Rotor speeds of 40,000–48,000 r.p.m. were used. The experiments at 0.91 and 0.45 mg ml⁻¹ were made using absorbance optics; the other experiments used interference optics. Both short (0.7 mm) and medium (3 mm) solution columns were used. The net charge on the peptide (Z) introduces some uncertainty into the results: the magnitude of the charge effect has been estimated from equations 62 and 63 of ref. 17, and is shown by the shaded areas on the graph. The known monomer M_r (including four Na⁺) is 1,676.

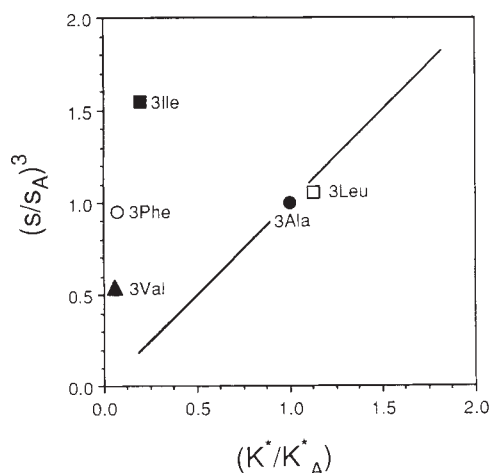


FIG. 3 Comparison of the effect of amino-acid substitution with the maximum effect predicted from the host-guest values of s (given in Table 1). Points on the line agree with the prediction from host-guest experiments, and points off the line show larger than expected changes in helicity as a result of making three substitutions (for example, Ala $\rightarrow$ Val) in the reference peptide. The peptide labelled 3Ala is the reference peptide and falls on the line by definition. The apparent two-state equilibrium constant K^* is $f/(1-f)$, where f is the fraction helix (Table 1) and K_A^* refers to the reference peptide 3Ala. For a two-state reaction, K^*/K_A^* is given by $(s/s_A)^3$ (ref. 18), where 3 is the number of substitutions of the chosen amino acid for Ala. Computations based on the Lifson-Roig¹⁹ theory of helix formation, in collaboration with work of J. A. Schellman, indicate that the two-state approximation gives an upper limit (data not shown) to the effect expected for an amino-acid substitution.

strongly to determining whether a given sequence will form an α -helix in a protein. Experiments with short dimeric coiled-coil helices show that helix-forming tendency varies widely among the 20 amino acids, and substitution of a single residue can have a marked effect on helix stability (W. F. DeGrado, personal communication), in agreement with the results reported here.

It is interesting to compare these measurements of helix-forming tendency with the helical preference P_α , given by the frequency of an amino acid in protein α -helices relative to its frequency in the whole protein. Data for P_α , taken from a study⁶ of protein X-ray structures, are given in Table 1. We were surprised to find that Phe (which is helix-destabilizing in our experiments) has the third highest P_α value of any amino acid. This indicates that P_α depends on other factors in addition to helix-forming tendency. It is likely that P_α depends also on the frequency that an amino acid occurs in a helix-helix or helix-sheet pairing site.

What determines the order of helix-forming tendency in non-polar amino acids? Hydrophobicity of the side chain cannot be the determining factor, because Ala and Leu have nearly equal helix-forming tendencies but very different hydrophobicities, and their helix-forming tendencies differ markedly from those of Val, Ile and Phe. Our finding that Val and Ile are helix-destabilizing is consistent with the view⁸, based on results for α -helix versus β -sheet formation by synthetic polypeptides in organic solvents, that β -branching causes an amino acid to be helix-destabilizing. Host-guest results⁵, which indicate that Ile is a better helix-former than Ala or Leu, have indicated that β -branching is only a minor factor in determining helix-forming tendency. Our results show further that Phe is helix-destabilizing. The side-chain rotamer conformations permitted in an α -helix are severely restricted by β -branching⁸, and the bulky aromatic ring of Phe also restricts the side-chain conformations that can occur in a helix⁸. Recent work has emphasized the energetic importance of the occurrence of particular side-chain rotamer conformations in the hydrophobic core of a protein⁹⁻¹². Our results indicate that restriction of side-chain rotamer conformations in the α -helix is similarly an important factor in determining the helix-forming tendency of a nonpolar amino acid.

Note added in proof: The distribution of side-chain chi 1 rotamer angles is known to be different in α -helices than in β -sheet or unordered segments of proteins (McGregor, M. J., Islam, S. A. and Sternberg, M. J. E., *J. molec. Biol.* **198**, 295–310 (1987)). For α -helices, the altered distribution of rotamer angles is, we suppose, correlated with a reduction in side-chain conformational entropy in the helix compared with the flexible chain.

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- Platzter, K. E. B., Ananthanarayanan, V. S., Andreotta, R. H. & Scheraga, H. A. *Macromolecules* **5**, 177–187 (1972).
- Marqusee, S., Robbins, V. H. & Baldwin, R. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5286–5290 (1989).
- Shoemaker, K. R. et al., *Proc. natn. Acad. Sci. U.S.A.* **82**, 2349–2353 (1985).
- Nelson, J. W. & Kallenbach, N. R. *Proteins* **1**, 211–217 (1988).
- Sueki, M., Lee, S., Powers, S. P., Denton, J. B., Konishi, Y. & Scheraga, H. A. *Macromolecules* **17**, 148–155 (1984).
- Williams, R. W., Chang, A., Juretic, D. & Loughran, S. *Biochim. Biophys. Acta* **916**, 200–204 (1987).
- Blout, E. R. in *Polyamino Acids, Polypeptides and Proteins* (ed. Stahmann, M. A.) 275–279 (University of Wisconsin Press, Madison, 1962).
- Piel, L., Nemethy, G. & Scheraga, H. A. *Biopolymers* **26**, 1273–1286 (1987).
- Ponder, J. W. & Richards, F. M. *J. molec. Biol.* **193**, 775–791 (1987).
- Lim, W. A. & Sauer, R. T. *Nature* **339**, 31–36 (1989).
- Sandberg, W. S. & Terwilliger, T. C. *Science* **245**, 54–57 (1989).
- Karpusas, M., Baase, W. A., Matsumura, M. & Matthews, B. W. *Proc. natn. Acad. Sci. U.S.A.* **86**, 8237–8241 (1989).
- Stewart, J. M. & Young, J. D. *Solid Phase Peptide Synthesis* (Pierce Chemical Company, Rockford, Illinois, 1984).
- Yphantis, D. A. *Biochemistry* **3**, 297–317 (1964).
- McMeekin, T. L. & Marshall, K. *Science* **116**, 142–143 (1952).
- Johnson, M. L., Correia, J. C., Yphantis, D. A. & Halvorson, H. R. *Biophys. J.* **36**, 575–588 (1981).
- Williams, J. W., Van Holde, K. E., Baldwin, R. L. & Fujita, H. *Chem. Rev.* **58**, 715–806 (1958).
- Strehlow, K. G. & Baldwin, R. L. *Biochemistry* **28**, 2130–2133 (1989).
- Lifson, S. and Roig, A. *J. Chem. Phys.* **34**, 1963–1974 (1961).

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Bioassays with a microphysiometer

J.C. Owicki and J.W. Parce

By sensitively monitoring the metabolic rates of living cells, a novel instrument detects cellular responses to stimuli as diverse as ligand-receptor binding and toxic chemicals.

In bioanalytical research, the two questions most commonly asked of a test compound are, "What is its binding specificity" and "What are its effects on a living system?" Binding is a matter of molecular structure, as commonly exploited in immunoassays and ligand-receptor binding assays. Biological effects deal primarily with function rather than structure; they are the domain of a wide variety of bioassays ranging from the triggering of the proliferation of cultured cells to toxicity in whole organisms.

Bioassays tend to be slower, more cumbersome, less precise, and more expensive than binding assays. For example, the standard assay for cellular proliferation, based on the incorporation of radiolabelled thymidine, typically takes several days¹. The Draize test for ocular irritancy requires seven days of somewhat subjective scoring of the pathological effects of compounds instilled into the eyes of rabbits². In contrast, binding assays are generally more suitable for automation in sample handling and data collection.

Microphysiometer device

We have devised an instrument called a silicon microphysiometer³ that is intended to overcome many of the drawbacks of bioassays. It can be used to detect a broad range of effects of agents on living cells in culture. Further, in some cases, it may permit assays that are, at present, performed on whole organisms or on isolated organs to be performed instead on cultured cells.

The microphysiometer works by detecting the effect of chemical agents to alter the metabolic rates of cells. Given the extensive interconnections among the biochemical reactions within a cell, a perturbation at one biochemical site will propagate, with attenuation or amplification, throughout the entire system. As intermediary metabolism is widely coupled to other cellular processes, for example through ATP production and consumption, it is a suitable system to monitor. Whether metabolic changes can be measured reliably depends on their size and kinetics compared to the sensitivity and time resolution of the detection device. In many cases, we have discovered that the detection of metabolic stimulation or inhibition using the microphysiometer is straightforward.

The principle of operation of the micro-

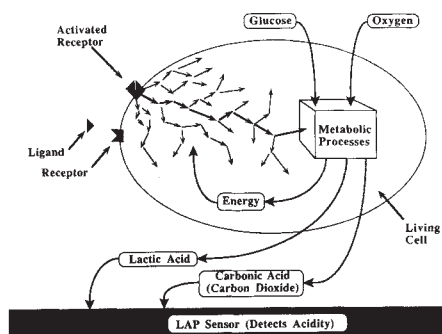


FIG. 1 Schematic diagram of relationships between cellular metabolic events and the LAP sensor.

physiometer is shown in Fig. 1. The main products of carbohydrate metabolism are acids that are excreted, acidifying the extracellular medium at a rate directly related to metabolic energy production. Cells are retained in a flow chamber (Fig. 2), and the rate of acidification during momentary cessations of the flow of culture medium is taken to be the metabolic rate.

The pH of the medium is measured with a light-addressable potentiometer sensor (LAPS)⁴, which is an integral part of the chamber. The LAPS is a silicon semiconductor device that functions by detecting the electrical surface potential at illuminated regions of the electrolyte-device interface. In this case, the surface contains proton binding hydroxyl and amino groups, and the surface potential is determined by the pH of the solution.

Adherent or nonadherent cells are retained in the flow chamber by a variety of methods⁵. Typical chamber volumes are a few microlitres, and measurements

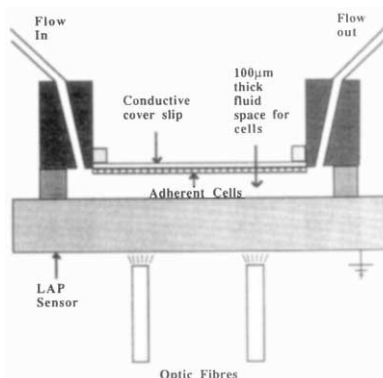


FIG. 2 Schematic cross-sectional diagram of the microphysiometer flow chamber.

of metabolic rate are performed in regions within the chamber containing about 10^3 cells in 100 nl. Acidification rates can be measured with a precision of a few per cent and with a time resolution as fine as about 30 seconds. Cells have been kept alive in the microphysiometer for as long as six days.

Additional components of the complete instrument include a pump for medium, a device for suppressing air bubbles in the narrow fluid stream, temperature regulation for the chamber, and electronics for the LAPS. The latter is on a circuit board in a personal computer that also controls fluid flow and data analysis.

Receptor activation studies

Figure 3 illustrates the ability of the microphysiometer to detect ligand-receptor interactions. Epidermal growth factor (EGF) is mitogenic towards human keratinocytes in culture⁷. When added to a culture of keratinocytes starved of macromolecular growth factors, it induces an increase in metabolic rate that peaks after about 15 minutes. This response occurs in the pharmacologically relevant

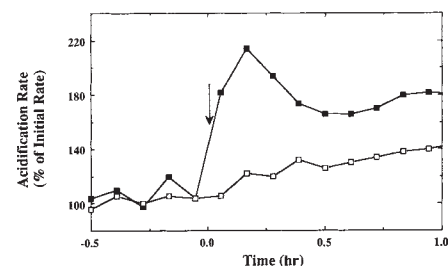


FIG. 3 Effect of epidermal growth factor (EGF) on the metabolic rate of keratinocytes. At the time indicated by the arrow, EGF (50 ng ml^{-1} ; solid squares) or control medium (open squares) was added to the microphysiometer flow chambers.

range of EGF concentrations and is inhibited by antibodies against EGF^{3,6}. Metabolic stimulation by receptor activation appears to be a rather general phenomenon, having also been for transforming growth factor alpha⁶, functionally transfected muscarinic acetylcholine and beta adrenergic receptors⁶, and prostaglandin E_1 ⁷. These receptors operate by a variety of signal-transduction mechanisms. Thus, the microphysiometer should be useful for a wide range of receptor-mediated bioassays, including those used in screening for new therapeutic drugs.

Toxicological applications

One toxicological application of the microphysiometer may be a replacement for the Draize test. In a small pilot study, we found an encouraging correlation between the *in vivo* ocular irritancy of compounds and their ability to inhibit the metabolism of cells in culture³. This result has been confirmed in a larger study⁸. Beyond being an accurate and fast (minutes to hours) method of detecting acute toxic insult, the technique can monitor another important toxicological parameter: the kinetics of recovery from insult (Fig. 4). It should be possible to

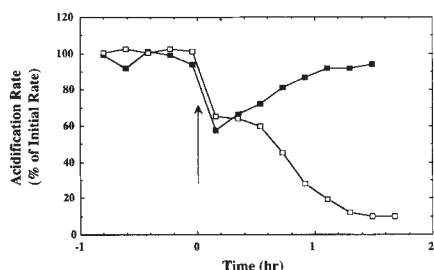


FIG. 4 Recovery of keratinocytes from metabolic inhibition caused by irritants. At the time indicated by the arrow, cells were exposed to dimethyl sulfoxide (DMSO; solid squares) or ethanol (open squares), each at a concentration sufficient to reduce the metabolic rate by 50 per cent after six minutes. During the two-hour period after the irritants were washed out, the cells recovered from exposure to the milder irritant (DMSO), but appeared to be terminally damaged by ethanol⁹.

detect the effects of toxic compounds on not only general metabolic rate, but also on the ability of cells to respond to the activation of specific physiological processes (for example, receptor activation).

Additional toxicological applications of the microphysiometer include tumour chemosensitivity testing and the detection of viral infection and of protection by antiviral drugs³.

Future prospects

The generality of using metabolic rates to monitor cellular responses permits a single instrument to be used for diverse applications. To ensure the specificity of the metabolic changes, it is necessary — and generally practical — that biological and chemical controls be designed into experiments.

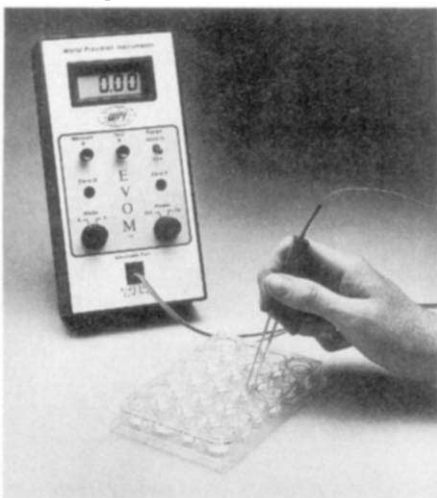
Other techniques that have been used to measure the metabolic rates of cultured cells include microcalorimetry¹⁰ and oximetry with a Clark oxygen electrode^{11,12}. We believe that the speed, sensitivity and convenience of the microphysiometer offer distinct advantages for many applications.

Molecular Devices Corporation is developing a commercial version of the microphysiometer for release in 1991.

Measure for measure

A set of 'chopstick' electrodes for measuring membrane potential, a portable pH meter and a pulsed electrochemical detector — measuring devices for improved accuracy.

THE latest STX-2 **chopstick electrodes** from World Precision Instruments are designed to facilitate the measurement of membrane potential and epithelial resistance when used in conjunction with the EVOM epithelial voltammeter (*Reader Service No. 101*). Each fixed pair of electrodes is 4-mm wide and 1-mm thick. The small size of the electrodes makes them ideal for placement in cell culture wells,

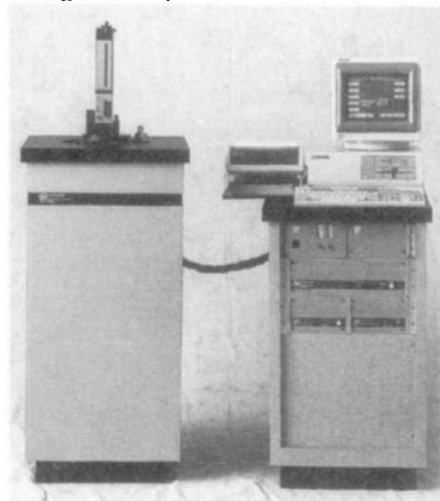


Measure membrane potential in wells with WPI's 'chopstick' electrodes.

particularly Costar Transwell culture cups, says WPI. The outer electrode is 2.5 mm longer than the other in order to fit into the deeper outer chamber of the well. Each prong has a silver/silver chloride pellet for measuring voltage and a silver electrode for passing current. When not in use, electrode tips can be stored dry or in electrolyte and can be sterilized with 70 per cent ethanol. The whole system, which includes the EVOM voltammeter and a pair of electrodes,

costs \$695 (US); replacement electrodes are \$100 (US) a set.

According to Quantum Design, MPMS₂ — the **SQUID-based variable-temperature magnetometer** — provides fast, precise measurements of the magnetic characteristics of materials over a broad temperature range (5 to 350 K) and applied magnetic fields ($\pm 10,000$ Oe) (*Reader Service No. 102*). The magnetometer uses a low T_c SQUID amplifier to measure magnetic moment over a dynamic range of 10^{-7} to 1.25 e.m.u. With a differential sensitivity of 10^{-8} , MPMS₂ can characterize samples in both thin film and bulk form, up to 8 mm in diameter and 7 mm in length. It measures the moment of a sample by moving it through a superconducting sensing coil at a fixed temperature and magnetic field. The gradient coil configuration produces a characteristic



Quantum Design's MPMS₂ magnetometer for magnetic moment analysis.

Later in 1990, a few prototype instruments will be placed in collaborating laboratories for technical evaluation. The LAPS device on which the microphysiometer is based is already part of a commercial product line called the Threshold system for detecting picogram quantities of generic DNA contaminants in recombinant pharmaceutical products¹³ and performing high-sensitivity immunoassays. □

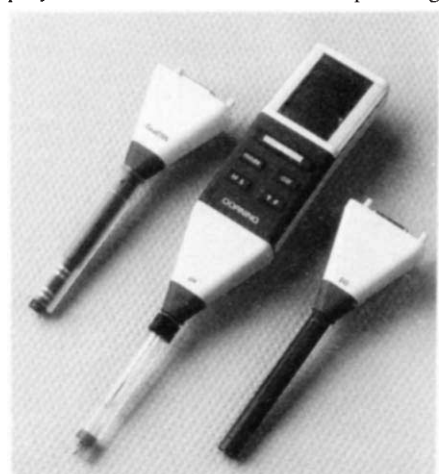
John C. Owicki is Director of Cell Research and J. Wallace Parce is Vice-President for Research at Molecular Devices Corporation, 4,700 Bohannon Drive, Menlo Park, California 94025, USA. For more information, fill in reader service number 100.

- Honegger, A. *et al.* *EMBO J.* **7**, 3045-3052 (1988).
- Draize, J.H., Woodward, G. & Calvery, H.O. *J. Pharmac. exp. Ther.* **82**, 377-390 (1944).
- Parce, J.W. *et al.* *Science* **246**, 243-247 (1989).
- Hafeman, D.G., Parce, J.W. & McConnell, H.M. *Science* **240**, 1182-1185 (1988).
- Shipley, G.D. & Pittelkow, M.R. *Arch. Dermatol.* **123**, 1541a-1544a (1987).
- Owicki, J.C. *et al.* (in preparation).
- Parce, J.W., Owicki, J.C. & Kercso, K.M. *Annls de Biologie Clinique* (in the press).
- Bruner, L.H. & Parker, R.D. *The Toxicologist* **10**, 258 (1990).
- Parce, J.W., Sigal, G.B., Kercso, K.M. & Owicki, J.C. in *ACS Symposium on Biosensors* (ed. Hatfield, W.E.) (in the press).
- Thermal and Energetics Studies of Cellular Biological Systems (ed. James, A.M.) (Wright, Bristol, 1987).
- Karube, I., Matsunaga, T. & Suzuki, S. *Analytica chim. Acta* **109**, 39-44 (1979).
- Li, X.-M., Liang, B.S. & Wang, H.Y. *Biotechnol. Bioengng* **31**, 250-256 (1988).
- Briggs, J. *et al.* *Am. Biotech. Lab.* **7**, 34-38 (1989).

three-peak signal and the magnetic moment is computed from the generated response curve. The \$59,950 (US) MPMS₂ can also be configured for resistivity and magnetoresistance (Hall Effect) measurements.

pH meters

Measure pH, conductivity and dissolved oxygen with the hand-held meter module and range of sensors called the Checkmate system from Ciba Corning (*Reader Service No. 103*). The battery-powered, shock-proof meter module features a large display that indicates results and operating



Ciba Corning's multipurpose meter.

parameters. To this are fitted one of three sensors. The pH sensor includes a replaceable ceramic reference junction and an ion barrier on the reference element that ensures high performance, says Ciba. The Checkmate system features auto-calibration, end-point sensing, five-measurement memory and 16-K ROM. Self-diagnostic functions include sensor replacement warning, low battery indicator, and a non-calibrated sensor warning.

If you are confused about which **pH electrode** is right for your laboratory requirements, then the Beckman *Guide to Futura Plus Electrodes, Buffers and Accessories* may provide some answers (*Reader Service No. 104*). Bulletin 7824 guides users through the process of electrode selection and provides information on how to optimize running conditions. It takes you through the most common choices: calomel or silver/silver chloride, electrode pair or combination, glass or epoxy body, refillable or gel-filled. Futura electrodes are available in four types: general purpose and biological combination electrodes, high-temperature combination electrodes, electrode pairs, and speciality combination electrodes. All electrodes feature a detachable connector/cable that can be used with all electrode types. It provides a tight seal and allows for quick interchangeability of electrodes.

Handy gadgets

According to Comark Electronics, its new 9003 **battery-operated thermometer** is water resistant and compatible with the company's full range of Type K thermocouple probes and sensors (*Reader Service*



Comark's battery-operated thermometer.

No. 105). The £125 (UK) 9003 portable thermometer is switchable between Celsius or Fahrenheit measurement. It has a measurement range of -100 to $+1,200$ °C and -150 to $+2,000$ °F with a resolution of one tenth of a degree up to $+1,000$ °C or $+1,000$ °F. The thermometer's display indicates the status of the battery, over-range measurements, and if there is an open circuit.

Kane-May Ltd is offering a **battery-powered pressure meter** and accessories in kit form (*Reader Service No. 106*). Depending on individual requirements regarding range and scale, Kane-May will supply the pressure test kit with any one of the company's pressure meters. The meters are available as three-scale or single-scale models, with standard or differential measurements. Scales available include psi, kPa, Pa, inches water gauge, mm water gauge, bar and mbar. The kit also includes tubing, a hand pump, connector and scale conversion chart and case.

Detection devices

Dionex has a new **pulsed electrochemical detector** (PED) that combines the detection techniques of conductivity and amperometry into one unit (*Reader Service No. 107*). These combined capabilities offer a powerful detection system for inorganic anions and cations, organic acids, amines, carbohydrates, oligosaccharides from glycoproteins, alcohols, aldehydes, thiols and sulphides. Electrochemical techniques are, says Dionex, often more sensitive and specific than UV detection or refractive index, particularly if the wavelength is less than 220 nm. PED is designed for methods development on any IC or HPLC system. Electronic pulsing in both conductivity and amperometry

modes virtually eliminates the noise, drift and electrode fouling associated with other electrochemical detectors, says Dionex. PED can be operated in series with a UV detector to provide much more information per analysis. Additional capabilities of PED include cyclic voltammetry, d.c. amperometry, pH and temperature.

Election microscopists may be interested in Voyager — the latest **X-ray microanalysis system** from Tracor Northern, which provides a new type of X-ray detector and a SPARC-based workstation from Sun Microsystems (*Reader Service No. 108*). The combination diamond window and germanium crystal Explorer X-ray detector provides, says Tracor, improved resolution and light element detection capabilities over an extended spectral range. The UNIX multitasking operating system offers windowing display capabilities so that the



Voyager provides X-ray microanalysis over an extended spectral range.

user has simultaneous onscreen control of all experimental and software operations. Data analysis software includes the Proza microvolume analysis program and Labnote for report generation. Prices for Voyager start at \$48,000 (US) not including the X-ray detector, which costs about \$24,000 (US).

Radiometer Analytical of Denmark is featuring the TraceLab system for **trace element analysis** down to and below the

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p.p.b. level (*Reader Service No. 109*). TraceLab, which is under computer control (IBM XT, AT or PS/2), comes with dedicated programs that guide the user through the analysis. Central to the TraceLab system, is the PSU20 potentiometer stripping unit, which controls the electrodes and is in command of the purge and stirring devices. During the stripping process, the PSU20 measures the poten-

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tial up to 30,000 times per second and counts how many times the potential stays within intervals of 2 mV. The PC identifies the metal present from the count versus potential data. Concentration of the metal is determined by integrating the counts of each peak. The SAM20 sample station, which includes electrodes, stirrer and beakers, completes the package.

Microplate readers

Read enzyme immunoassay (EIA) microplates in any location with the CLS 963 **portable photometer** from Quatro Biosystems (*Reader Service No. 110*). The 220 × 110 × 270 mm CLS 963 is equipped with ten interchangeable filters and has a 10-nm bandwidth for measurement over the 340-690-nm wavelength range. The instrument is designed to be blanked on air or on solutions of up to 0.3 absorbance units. The CLS 963 can be mains or battery-operated and can be linked to an optional IBM PC or compatible computer, or 40-column thermal printer via a RS232 interface. Datasure custom EIA software is also available.

SOFTmax 2.01 **software**, now available on the Macintosh from Molecular Devices, offers full control of microplate readers and data analysis (*Reader Service No. 111*). Using SOFTmax 2.01, users can set up routine methods in files, including information on instrument settings, templates, data analysis and display options. Dilution series for unknown or controls and concentration series for standards can be set up, and outlying wells can be masked from data analysis. Read



SOFTmax: microplate analysis software.

modes include single or dual-wavelength kinetics with read intervals that can be set as short as five seconds and run times as long as 24 hours. The software allows the user simultaneously to display real-time kinetic reactions in all 96 wells with either a positive or minus change in absorbance. In addition, the user can analyse non-linear kinetics, zoom in on a particular well, calculate V_{max} and plot V_{max} rate or time to V_{max} against concentration. SOFTmax offers a choice of six curve-fitting methods.

Weights and measures

Ohaus has introduced five new models in the CT Series of **portable electronic balances**, which have capacities ranging from 10 to 6,000 g (*Reader Service No. 112*). The CT Series balances are available with capacities of 10 g, 200 g, 600 g, 1,200 g and 6,000 g and cost between £295 and £425 (UK). All are supplied with auto-calibration and a precise calibration mass. Users can choose to weigh in grams, ounces, troy ounces, pennyweights, carats or pounds, as well as parts counting mode. Each balance can be mains or battery-operated. For complete portability, the balance can be powered by eight alkaline batteries, which, Ohaus says, should provide 100 hours of continuous use. Additional features include an automatic switch-off facility for battery conservation, high-speed LCD, and an optional RS232 interface.

The Vibrasorb **vibration damping mount** allows vibration-free weighing on sensitive analytical balances, says R.B.



Radley's vibration damping mount.

Radley & Company (*Reader Service No. 113*). According to the company, Vibrasorb can absorb vibrations generated by nearby pumps, stirrers and other laboratory equipment down to 9 Hz. The balance table has a 450 × 560 mm surface area and is made of solid, dense Terrazzo with a black and white scratch-resistant top. It weighs 39 kg and is designed to support weights up to 16 kg. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

CORRECTION A product review for Lagan, Inc. (*Nature* **343**, 491; 1 February 1990) incorrectly stated that "the probes individually identify different sequences that are repeated 300 to 700 times throughout the human haploid genome". It should have read "300 to 7,000 times".